Developmental and functional characterization of cystatin and chitinase of *Acanthocheilonema viteae*

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SUMMARY

Nematodes which cause filariasis have detrimental effect on humans. Strategies to eliminate this disease are based on mass treatment with drugs like ivermectin. However, they have several drawbacks such as long duration required for treatment and tenuous financial supports. Understanding the molecular mechanisms of genes required for parasitism will help to develop novel therapeutic and preventive strategies.

The aim of this study was a detailed functional characterization of cystatin and chitinase of *Acanthocheilonema viteae,* a rodent filarial nematode. To this end, *C. elegans* was used as a heterologous system to determine the spatial expression pattern of *A. viteae* cystatin and chitinase and thereby their possible functions. The promoter of cystatin drove the expression of reporter, GFP, to the pharyngeal and rectal gland cells of transgenic *C. elegans* lines, this being compatible with the fact that cystatin is secreted in the parasites. This also suggests that cystatin in *A. viteae* is probably required for moulting since generally in *C. elegans* the enzymes required for moulting are stored in the pharyngeal gland cells. Moreover, knockdown of cystatin by RNAi delayed moulting of the infective L3 to the L4. However, the RNAi effect was transient and the delay in moulting did not affect the viability and infectivity of the larvae. Analyses of the developmental regulation of cystatin by real-time PCR showed that it is maximally expressed in the blood microfilarial stage, which are exposed to the full force of host immune responses. This is compatible to the fact that *A. viteae* cystatin immunomodulates the host immune responses.

This study also determined that chitinase of *A. viteae* plays an essential role in the moulting of the L3 larvae since knockdown of chitinase inhibited moulting in 90% of the L3 larvae thereby killing them. Moreover, maximum expression of chitinase was observed by real-time PCR in the L3 stage supplementing that it is involved in moulting. RNAi of chitinase in adults led to the release of unhatched microfilariae confirming the essential catalytic role of chitinase in the degradation of the chitin egg shells. This study substantiates that cystatin and chitinase of *A. viteae* are attractive intervention targets due to their essential endogenous functions in the parasite.

ZUSAMMENFASSUNG

Die Infektion mit Filarien (Nematoden) ruft massive Schädigungen beim Menschen hervor. Strategien zur Bekämpfung dieser Parasitose basieren auf einer Massenbehandlung mit Ivermectin und Derivaten. Allerdings ist die Behandlung der Patienten zeitaufwändig und teuer. In diesem Zusammenhang stellt Aufklärung molekularer Mechanismen, die die parasitische Lebensform dieser Würmer ermöglichen, einen neuen Ansatzpunkt für die Entwicklung von Therapeutika und Präventivmaßnahmen dar. Die Moleküle Cystatin und Chitinase wurden, auf Grund ihrer immunodulatorischen und katalytischen Eigenschaften, bei der Nager-Filarie *Acanthocheilonema viteae* als essentielle Proteine identifiziert. Ziel der vorliegenden Arbeit war daher die detaillierte, funktionale Charakterisierung dieser beiden Proteine.

Um das räumliche Expressionsmuster und damit potentielle Funktionen des Sekretionsprotein Cystatins zu ermitteln, wurde der frei lebende Nematode *Caenorhabditis elegans* als heterologes Expressionssystem genutzt. Unter dem Einfluss des Cystatin-Promoters konnte GFP in pharyngealen und rektalen Zellen von *C. elegans* exprimiert werden. Möglicherweise ist das Cystatin damit bei *A. viteae* in den Häutungsprozess involviert, da bei *C. elegans* derartige Enzyme in den pharyngealen Zellen gespeichert werden. Des Weiteren wurde der Häutungsprozess der infektiösen L3 zum Stadium der L4 durch Ausschalten des Gens mittels RNAi um drei Tage verzögert. Allerdings war der Effekt transient und die Verzögerung des Häutungsprozesses beeinflusste weder die Viabilität noch die Infektiösität der Larven. Die Analyse der Regulation von Cystatin während der Entwicklung des Parasiten mittels der Real-Time PCR zeigte, dass das Gen im Stadium der Mikrofilarien, die der Immunantwort des Wirtes voll exponiert sind, maximal exprimiert wird.

Für die Chitinase von *A. viteae* konnte eine essentielle Rolle im Häutungsprozess nachgewiesen werden. Das Ausschalten des Gens führte zu einer Hemmung der Häutung bei 90 % der L3 und damit zu ihrem Tod. Die maximale Expression im L3 Stadium des Parasiten ist ein weiterer Hinweis darauf, dass dieses Protein in den Häutungsprozess involviert ist. Mittels RNAi bei adulten Parasiten konnte die katalytische Rolle der Chitinase beim Abbau des Chitins im Ei bestätigt werden, da hier nur ungeschlüpfte Mikrofilarien ausgeschieden wurden.

Die Ergebnisse dieser Arbeit liefern weitere Hinweise darauf, dass sowohl das Cystatin als auch die Chitinase von *A. viteae* auf Grund ihrer essentiellen endogenen Funtionen attraktive Zielmoleküle in der Bekämpfung von Filariosen darstellen.

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1. INTRODUCTION

1.1 Filariae

1.1.1 Filarial diseases in humans: Epidemiology, clinical manifestations and treatment

Infections with filarial nematodes are a major problem of public health in tropical countries and affect about 120-150 million people (http://www.who.int/inf-fs/en/fact102.html). The major forms of filariasis are; lymphatic filariasis, caused by *Wuchereria bancrofti, Brugia malayi* and *Brugia timori*, onchocerciasis or river blindness caused by *Onchocerca volvulus* and loiasis caused by *Loa loa* [1]. The filarial nematodes belong to the superfamily *Filarioidea* in the phylum *Nematoda* (Fig.1.1).

Phylum	Class - Subclass	Order - Superfamily - Family - Subfamily	Genus
Nematoda	Chromadorea - Rhaditia	Spirurida - Dracunculoidea	*Dracunculus*
		- Oxyuroidea	*Oxyuris*
		- Filarioidea - Filariidae	*Filaria, Parafilaria*
		- Onchocercidae - Dirofilariinae	*Dirofilaria, Loa*
		- Onchocerciinae	*Onchocerca, Litomosoides, Brugia Acanthocheilonema Wuchereria*
		- Ascaridina	*Ascaris*
		- Rhabditida	*Caenorhabditis*

Figure 1.1 Classification of the filarial nematodes.

Adapted from 'Allgemeine Parasitologie', 2005 by Hiepe T, Lucius R and Gottstein B.

Onchocerciasis or river blindness occurs in 34 countries in Africa, Latin America, and the Arabian Peninsula. An estimated 37 million persons are infected with *O. volvulus* the vast majority of who live in Africa. The infection has caused blindness in 270,000 and left another 500,000 with severe visual impairment [2]. More than a blinding disease, onchocerciasis is a chronic systemic illness capable of causing extensive and disfiguring skin changes, musculoskeletal complaints, weight loss, changes in the immune system, and perhaps epilepsy and growth arrest. The disease, which is endemic in some of the world's poorest areas, has had a major impact on the economic and social fabric of communities [3].

Infective L3 larvae of *O. volvulus* enter the human during the blood meal of an infected female *Simulium* fly. Within 1-3 months the L3 larvae moult to the L4 and adult male and female worms which dwell in nodules. Each female worm releases 1300-1900 microfilariae per day for 9-11 years [4]. The microfilariae are ingested by *Simulium* flies and develop to the L2 and subsequently to the infective L3 stage. The manifestations of onchocerciasis in humans are almost entirely due to the localised host inflammatory responses to dead or dying microfilariae. Microfilariae enter the cornea from the skin and conjunctiva, and a punctuate keratitis develops around dead micorfilariae which clears when inflammation settles. With exposure to years of heavy and prolonged infection, sclerosing keratitis and iridocyclities are likely to develop, causing permanent visual impairment or blindness [5]. Onchodermatitis, chronic and acute papular, is also one of the consequences of the disease.

Strategies to eliminate onchocerciasis have, in the past, been based on vector control and/or mass treatment with the microfilaricidal drug ivermectin [6]. Ivermectin is used in WHO–sponsored multinational health programs. The drug acts as an agonist of the parasite neurotransmitter, γ-aminobutyric acid [7], and by inducing an influx of Cl^- through channels not regulated by γ-aminobutyric acid. Ivermecitin is an efficient microfilaricidal and though it does not kill adult worms it temporarily affects the embryonic development and release of microfilariae from the female adult worms [8]. However there are several drawbacks to the chemotherapeutic treatment [2]. For instance it, (i) cannot achieve eradication of parasite infection unless treatment is sustained for 15 - 20 years i. e., throughout the full length of life of the adult worm, and (ii) have tenuous financial support. A more efficient treatment and control method

has been suggested, which would use drug treatment combined with a prophylactic vaccine. Therefore, it is important to characterise antigens playing critical roles in parasite development and transmission processes. One of the significant experimental limitations that have hindered the functional characterisation of chemotherapeutically and immunologically relevant target molecules of *Onchocerca* species is that the parasite is strictly primate specific [17]. To circumvent this problem the proxi-model of the rodent filarial nematode *Acanthocheilonema viteae* in the jird *Meriones unguiculatus* was used for this study (Fig 1.2).

Figure 1.2 A larva of *Acanthocheilonema viteae* attacked by macrophages.

Source: Archive of the Dept. of Molecular Parasitology, Humboldt University Berlin.

1.1.2 The rodent filaria *Acanthocheilonema viteae* as a proxi model

Research in filariasis is dependent on the use of laboratory animal models owing to the inability to measure adult parasite population in humans. The filarial parasites are host-specific and so the first type of model system involves the use of parasites in surrogate models. The *Brugia spp.* / BALB/c mouse system has been used as a chemotherapeutic [9] and immunological [10] model for the brugian filariasis. A similar approach with *Onchocerca spp.* involves the implantation of *Onchocerca spp.* in subcutaneous chambers in CBA/J or DBA/2J mice [11, 12]. These systems have a set back in that they rely on studies of a parasite in its non-

natural host, a disadvantage that can be overcome by the use of full life-cycle models of the the filaria *Brugia pahangi* in cats [13], bovine *Onchocerca spp*. or rodent filariae, like *Acanthocheilonema viteae* (Fig. 1.3) and *Litomosoides sigmodontis,* in their natural hosts [17]. Owing to the lack of adequate immunoreagents the underlying immunological mechanisms can however not be investigated in these model systems. The *B. pahangi* / cat model serves as a model for *Brugia* and *Wuchereria* in humans [13]. This model system can be used to elucidate relationships between infection, immunity and disease states in lymphatic filariasis.

A. viteae in its natural host *Meriones unguiculatus* serves as a model for onchocerciasis, the disease caused by *O. volvulus*. This filarial model has some parallels to *O. volvulus* in that both reside in the subcutaneous tissue of their hosts and are therefore in the same immunological compartment. However, they do not form nodules and eye lesions like *O. volvulus*. Furthermore, both parasites share an array of antigenic similarities as demonstrated by anti-*O. volvulus* monoclonal antibodies [14], and a high homology in corresponding molecules that have so far been characterised from both parasites. In addition, cross-protection between species has been shown in filariasis [15, 16] so that vaccine candidates established in one system could be tested in others. The *A. viteae* / *Meriones* system allows the study of resistance to challenge infection following immunisation [17].

In the *A. viteae* / jird-model, it has been shown that immunisation with irradiated *A. viteae* L3 led to 90% protection against challenge infection, while immunisation with excretory-secretory products (ESP) led to 70% protection [18]. Parallel results were also obtained using irradiated L3s in other filariasis models [19, 20, 21, 22] and it could be shown in this model that immunisation with irradiated L3 could also lead to resistance against homologous challenge infection [17]. Therefore, the *A. viteae* / *Meriones* system was used in this study.

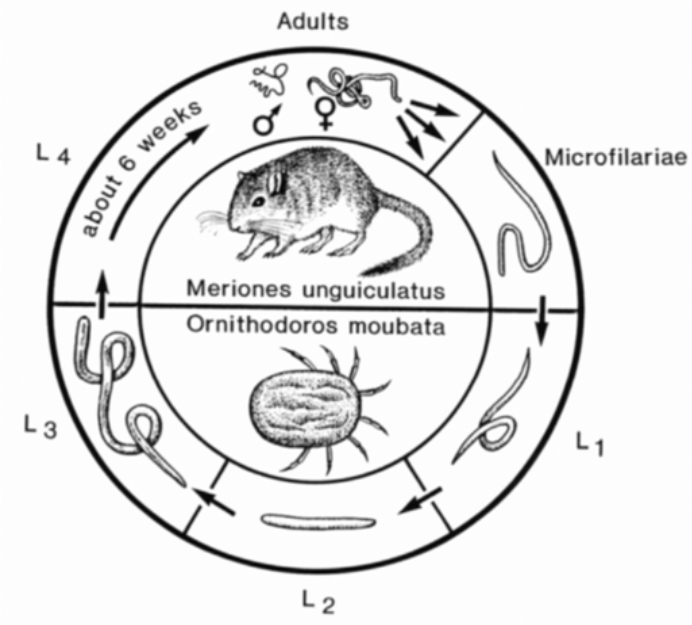

Figure 1.3 The life cycle of *Acanthocheilonema viteae*

The microfilariae are taken up by the arthropod host during the blood meal and develop to the L2 and L3 stages. The infective L3 are transmitted to the rodent host where they develop to adults, which release microfilariae.

1.2 Target molecules for intervention in *A. viteae*

The search for chemotherapeutically and immunologically relevant target molecules in *A. viteae* led, among others, to cystatin (cysteine protease inhibitor) and chitinase (an enzymatically active molecule).

1.2.1 Cystatin

The cystatin superfamily consists of evolutionarily related reversibly, tightly binding inhibitors of cysteine proteases [23]. Based on their amino acid sequences, cysteine protease inhibitors can be assigned to three major families. Family 1 (Stefins) comprises unglycosylated inhibitors of about 11 kDa (Cystatin A and B) that are devoid of a signal sequence and disulfide bonds. Cysteine protease inhibitors of family 2 (Cystatins) exhibit molecular masses of about 13 – 14 kDa and contain a signal sequence plus 2 disulfide bonds in proximity to the carboxy terminus. Family 2 cystatins, human cystatin C, D, S, SA and SN, are mainly exported out of the cell and are thus present in most biological fluids. Cystain C plays a role in the control of invariant chain degradation and antigen presentation [24]. Cystatin D, S, SA and SN play a role in the protection of the oral cavity and eyes against proteolytic activities of

cysteine proteases of inflammatory cells, viruses or bacteria [25]. Human cystatin F occurs in human natural killer cells, in dentritic cells (DC's) and in murine T cells [26]. Family 3 of the cystatin superfamily (Kininogens) contains 3 cystatin-like domains, disulfide bonds and carbohydrate groups.

Cystatins blocks the active centre of the protease by forming a tight, equimolar complex with the latter [23]. Three highly conserved reactive domains are involved in the interaction between the protease and inhibitor: (i) N terminal domain around Gly11 that interacts with substrate-binding domains S3, S2 and S1 [27, 28], (ii) hairpin loop between Gln53 and Gly57 (QVVAG region) [29] and (iii) hairpin loop containing the conserved Pro103 and Trp104 [29]. Nematode cystatins have been characterised regarding their capacity to inhibit the activity of cysteine proteases. Cystatins of *O. volvulus*, *Nippostrongylus brasiliensis* [30] and *Haemonchus contortus* [31] have been described to inhibit cysteine proteases cathepsin L and S, which are involved in the proteolytic processing of polypeptides, more efficiently than cathepsin B. Moreover, cystatins of *B. malayi* [32], *O. volvulus* and *L. sigmodontis* inhibit legumains or asparaginyl endopeptidases (AEP).

Studies of *O. volvulus* [33], *B. malayi* [32] and *L. sigmodontis* [34] have determined that cystatins are immunomodulators of host responses. They interfere with the antigen specific T cell responses by suppressing antigen presenting cells (APCs) to process antigen, generate and load MHC class II molecules and subsequently stimulate T-cell responses. *O. volvulus* cystatin significantly reduces the proliferation of human peripheral blood mononuclear cells (PBMC) triggered with purified protein derivative [33]. Recombinant cystatin of *N. brasiliensis* was shown to inhibit antigen processing of ovalbumin by lysosomal cysteine proteases from the spleen of mice [30]. Therefore, cystatins of parasitic nematode are regulatory proteins with a capacitiy to modulate APC, mostly macrophages, activities that in turn influence downstream immune reactions [35]. Nematode cystatins also have a profound effect on the production of cytokines. *O. volvulus* cystatin induces an early TNF-α (Tumor Necrosis Factor-α) response in human PBMC followed by a down regulation of IL-12 production and a massive increase in IL-10 production [33]. Cystatins of nematodes, regardless of whether they are parasitic or free-living, share with other members of the cystatin superfamily the potential to upregulate the NO

production of IFN-γ (Interferon-γ) activated macrophages [36, 37, 38]. Moreover, *A. viteae* cystatin has been shown to function as an allergen [39].

1.2.2 Chitinase

Chitinases (EC 3.2.1.14) are enzymes that break down chitin, a homopolymer of N-acetylglucosamine (GlcNAc), to its monomers. They are ubiquitous in the plant and animal kingdom [40] and play important structural, physiological, metabolic and defensive roles [41, 42]. Chitinases are glycosyl hydrolases, a family that is subdivided according to their hydrolysis mechanisms and amino acid sequence similarities of catalytic domains [43, 44, 45] (http://afmb.cnrs-mrs.fr/CAZY). Chitinases belong to class 18 and 19 of this grouping. Family 18 chitinases are endochitinases that cleave chitin by a retaining mechanism through which the beta-linked polymer is cleaved to release beta anomer products. Family 19 chitinases are mostly found in plants, some bacteria and nematodes and cleave chitin by an inversion or retention mechanism [46, 47, 48]. Family 19 class IA/I and IB/II enzymes differ in the presence (IA/I) or absence (IB/II) of an N-terminal chitin-binding domain. Chitinases also have a carbohydrate-binding module that promotes binding of the enzyme to insoluble chitin. The chitin binding domain of filarial family 18 chitinases (EC 3.2.1.14) are in the carbohydrate-binding module family 14, and contain six conserved cysteins that probably form three disulfide bridges [44, 45] (http://afmb.cnrs-mrs.fr/CAZY/).

Chitinases have been described for the infective larval (L3) and adult stages of *A. viteae* [49, 50] and *O. volvulus* [50], as well as in the microfilarial stage of *B. malayi*, *B. pahangi* [51] and in infective larvae (L3) and microfilariae of *W. bancrofti* [52]. L3 chitinases of *A. viteae* and *O. volvulus* are accumulated in the glandular oesophagus of the worms in their insect vectors, and released when the larvae are transmitted to the hosts and eventually diminish following moulting from L3 to L4 [50]. Microfilaria-specific chitinases [51] could be recognised by a monoclonal antibody in extracts of *B. malayi* microfilariae following several days of maturation in the vertebrate host as blood-borne microfilariae [53]. Interestingly, the appearance of these chitinases corresponds with the parasite's ability to infect the insect host [51].

The presence of chitin, a substrate of chitinases, has been biochemically documented in various nematode species and tissues. Chitin has been shown to be a component of the egg shells of many nematodes including the filariae [54]. In addition, chitin has been demonstrated on the sheath of *B. malayi* microfilariae [55] and *W. bancrofti* microfilariae [56]. Chitin has also been detected ultrastructurally in the pharyngeal cuticle of plant parasitic nematodes [57]. Chitinases are extracellular proteins, and while presumably they have a biological role in egg hatching, the existence of multiple genes and stage-specific expression indicates chitinases may have other functional roles in the nematode life cycle [58].

1.3 *Caenorhabditis elegans* as a heterlogous system to study cystatin and chitinase of *A. viteae*

The free-living nematode *Caenorhabditis elegans* is being used increasingly as a model system for parasitic nematodes where defining the function of genes of interest can be difficult due to lack of appropriate knock-out approaches or suitable functional assays. *C. elegans* offers a series of features that have made it a model system of parasites [59, 60]. The genetic and physical maps of *C. elegans* six chromosomes have been constructed and the sequence of the entire genome has been determined and annotated (www.wormbase.org). Phylogenetic analysis groups place *C. elegans* in Clade V along with the parasitic strongylid nematodes such as *Haemonchus contortus, Nippostrongylus brasiliensis* and *Necator* and *Ancylostoma* spp [61]. *C. elegans* not only displays morphological similarities but also shares processes and characteristics with parasitic nematodes, including the presence of a protective cuticle, dauer stages, biochemical adaptations to extreme conditions, moulting and reproduction. Nematode genome projects and Expressed Sequence Tag (EST) analyses have already identified parasite genes homologous to many *C. elegans* genes [62, 63]. Use of *C. elegans* as a heterologous system for studying gene function and regulation is important to fully exploit the parasite sequence data and to further the understanding of parasite biology [64, 65, 66, 67].

The life cycle of *C. elegans* is rapid, taking just 3.5 days at 20°C (Fig. 1.3). Embryogenesis, which involves development from fertilization to hatching, generates the first larval stage. Post-embryonic development involves growth through four larval

stages (L1 to L4) before the final moult to produce the adult. In the absence of food and at high population density, an alternative stage, the dauer, is formed at the second moult instead of the normal L3. This stage in *C. elegans* is similar to the microfilarial and L3 stage of parasitic nematodes, which also can survive up to months in the hosts.

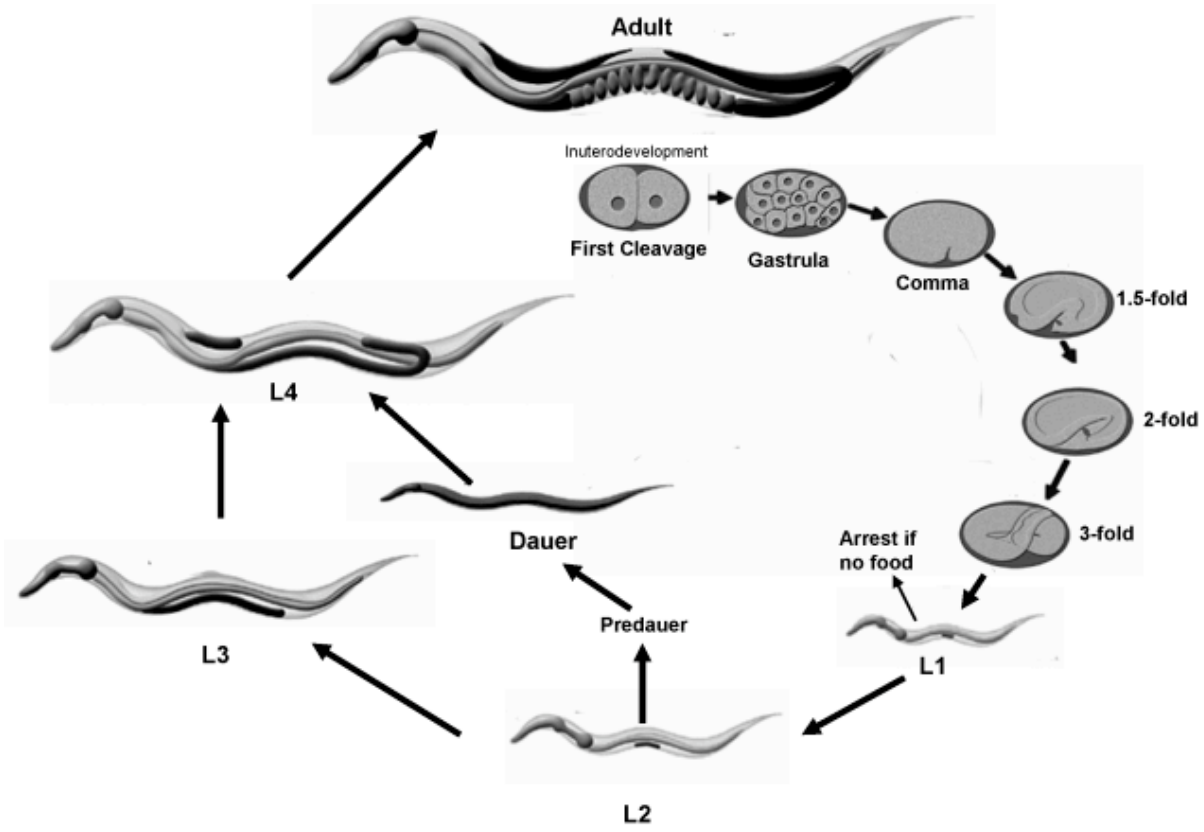

Figure 1.4 Life cycle of *Caenorhabditis elegans* at 20°C

Source: www.wormatlas.org

C. elegans has been successfully used as a heterologous transformation system to study the function of parasitic nematode genes by investigating the expression of the parasite promoters in *C. elegans*. The conservation of regulatory elements and transcription factors across widely divergent groups [68] suggests that, for many genes, there is likely to be sufficient conservation of regulatory mechanisms between *C. elegans* and parasitic nematodes to detect promoter function using this heterologous system. *C. elegans* are transformed with constructs containing parasite gene promoters. Efficient transformation is achieved by injection of DNA into the syncytial gonad arms [69]. To examine expression patterns, genomic DNA from promoter regions is cloned next to suitable reporter genes such as green fluorescent

protein (GFP) [70, 71]. Promoters of various parasites have been studied successfully to determine the spatial and temporal expression pattern of genes of parasites [65, 72, 73, 74].

C. elegans is also used as a heterologous expression system for parasite proteins [74]. Various studies have been done to identify potential target antigens for use in vaccination or as novel drug targets for helminth control. Although protection has been reported following vaccination with a number of native antigens isolated from parasite extracts [75], development of these for practical use in the field is difficult. However, the recombinant parasite proteins expressed in *E. coli* offered limited protection. This may be due to glycosylation and/or conformation differences between bacterial expressed and native proteins. Therefore, *C. elegans* is an alternative expression system to express parasite proteins in a similar form to native proteins. Moreover, expression in *C. elegans* would also ensure that the protein is functionally active [76].

1.4 Functional analyses by RNA interference

RNA interference (RNAi) is a gene silencing mechanism first characterised in *C. elegans* [77] and similar to the post-transcriptional gene silencing previously described in plants. This mechanism of gene silencing is effective in a diverse range of organisms including insects, planarians, protozoans and in mammalian cells [78, 79, 80, 81]. The development of RNAi technology for parasitic nematodes is a crucial step in elucidating gene functions. RNAi uses the endogenous cellular machinery that plays roles in gene regulation [82] and anti-viral / antitransposon defense mechanisms [83]. Effective gene silencing can be achieved by injection of double stranded RNA (dsRNA) into the adult worm body, by soaking any life-cycle stage in dsRNA, by feeding worms on *E. coli* expressing dsRNA or by transformation of adult worms with a dsRNA-expressing plasmid [77, 84, 85, 86].

1.4.1 The RNA interference mechanism

The process involves the dsRNA trigger being processed into small interfering (si) RNAs by a complex containing the ribonuclease (Dicer), the dsRNA binding protein RDE-4, the Argonaute related protein RDE-1 and the Drh-1 helicase [87] (Fig.

1.4). All four of these proteins are essential for the initiation of RNAi but not for its maintenance. The RNA-dependent RNA polymerases (RdRP), RRF-1 and EGO-1, are essential for amplification of the response in somatic and germline tissues, respectively. The siRNAs produced by the Dicer complex acts as primers using the target mRNA as a template. As a result, dsRNA is produced again, which then serves as substrate for Dicer. In *C. elegans*, RNAi is systemic in nature and siRNAs are transmitted systemically by interacting with the SID (systemic RNAi defective) protein. The SID protein is expressed in cells with direct environmental contact [88]. It functions by facilitating passive cellular uptake of dsRNA and longer dsRNAs are transported into the cells more efficiently.

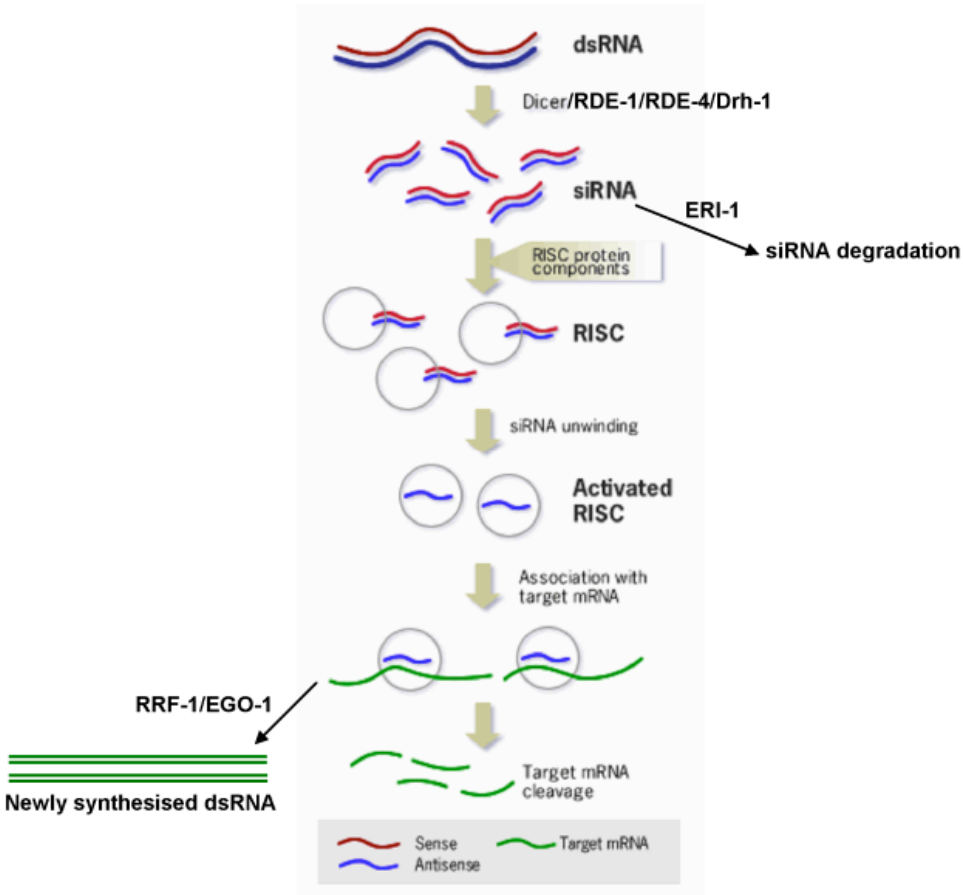

Figure 1.5 Model for RNA interference (RNAi) pathway in *Caenorhabditis elegans*.

Dicer: Ribonuclease, RDE: RNAi deficient, siRNA: small interfering RNAs, RISC: RNA induced silencing complex, RRF and EGO: RNA dependent RNA polymerases. Adapted from www.ambion.com/RNAi.

1.4.2 RNAi in parasitic nematodes

The effectiveness with which dsRNA can specifically reduce or ablate mRNA transcripts in *C. elegans* led to the extension of this technology to various other organisms including parasitic nematodes. In *N. brasiliensis*, soaking with dsRNA of acetylcholinesterase (AchE B) led to a reduction in acetylcholinesterase activity [89]. In *Meloloidogyne incognita* incubation in dsRNA to a dual oxidase enzyme led to a decrease in the number of female worms and in egg production [90]. Several genes have also been targeted by RNAi in the adult stage of *B. malayi*, with specific decreases in mRNA level and lethal or abnormal microfilarial phenotypes observed [91]. RNAi targeting of cysteine protease genes led to reduced moulting in *O. volvulus* L3 stages [92]. A reduction in moulting was also observed following soaking of *Ascaris suum* L3 stage larvae in dsRNA to pyrophosphatase [93].

RNAi effects are also reported in Trichostrongyloid nematodes of veterinary importance. In *T. colubriformis*, feeding of L1 through to L3 stage larvae on bacteria expressing dsRNA to tropomyosin resulted in delayed development to the L3 stage [94]. Moreover, when ubiquitin dsRNA was delivered by electroporation delayed larval development and death were observed. In *H. contortus*, a significant decrease in specific transcript level was observed following soaking of L3 and adult stages in dsRNA to beta-tubulin [95]. These studies suggest that an RNAi pathway exists in parasitic nematodes and that some genes can be silenced by dsRNA soaking. Preliminary studies have shown that the genes involved in RNAi such as *dcr-1, rde-1, drh-1, ego-1* and *rrf-1* have been identified in *H. contortus* (Saunders G, Gilleard JS and Britton C, unpublished data). In contrast, other genes such as *sid-2* and *rsd* essential for siRNA production and systemic RNAi in *C. elegans* do not seem to be conserved in *H. contortus*. These results suggest that with optimisation of conditions for either soaking or electroporation it may be possible to specifically reduce transcript levels for parasitic nematode genes and thereby analyse their physiological functions.

2 GOALS OF THIS STUDY

Filarial diseases caused by parasitic nematodes are a major problem of public health in tropical countries. Understanding the physiological functions of proteins required for parasitism will help to develop novel therapeutic and preventive strategies. The objective of this study was to characterize the proteins cystatin and chitinase, which are attractive intervention targets, and to investigate their physiological functions in *Acanthocheilonema viteae*, a rodent filarial nematode.

The genomic and promoter sequences of cystatin and chitinase were analysed to determine their spatial expression pattern. To this end, the free living nematode, *Caenorhabditis elegans* was used as a heterologous system to study the expression pattern. *C. elegans* was also used an expression system, to obtain *A. viteae* proteins with nematode specific post-translational modifications.

The physiological functions of cystatin and chitinase in *A. viteae* were also analysed. The reverse genetics method of RNA interference was used to knockdown cystatin and chitinase at the larval and adult stages. This study determined the essential roles of these proteins in the development of the parasite.

In addition to the determination of spatial expression pattern of cystatin and chitinase the temporal expression and regulation of these proteins during the developmental life cycle of *A. viteae* were determined. Finally, the potential of cystatin as a vaccine was analysed by immunization studies in the host *M. unguiculatus.* To this end, cystatin was used for immunization as protein and DNA vaccines.

3 RESULTS

3.1 *C. elegans*: A heterologous system for promoter studies and expression of cystatin and chitinase of *A. viteae*

Cystatin and chitinase are suggested to be attractive targets of intervention in the filarial nematode, *A. viteae.* These proteins are suggested to play many essential roles in the developmental cycle. In order to understand the functions of cystatin and chitinase in *A. viteae,* it was essential to determine the promoters and the factors involved in the expression of these genes. However, functional analysis of *A. viteae* promoter regions is still limited by the absence of nematode cell lines or even a suitable parasitic nematode model. This, however, can be overcome by using the model nematode *C. elegans* as there is only limited data on transformation of parasitic nematodes. In this section, the organisation of the promoter and genomic sequences of *A. viteae* cystatin and chitinase is described. The spatial expression pattern of cystatin and chitinase was determined in transgenic *C. elegans* lines. Furthermore, expression of cystatin in *C. elegans* was attempted to evaluate its potential as a vector for expression of *A. viteae* candidate vaccine antigens.

3.1.1 Promoter and genomic sequences of A. viteae cystatin and chitinase

A prerequisite to the determination of the complete genomic sequence of *A. viteae* cystatin was the isolation of clonal genomic recombinants containing the complete gene. An *A. viteae* genomic library (provided by Dr. Jörg Hirzmann, University of Gießen, Germany), constructed in λ dash II using genomic DNA from a mixed population of adult worms, was used to screen for cystatin genomic clones. The screening was done by plaque hybridisation using a DIG-labelled N-terminal cDNA sequence corresponding to nucleotides 89 to 493 bp of the cDNA sequence of cystatin (Hartmann et al., Acc. No: L43053). After screening of ~10,000 plaques, a positive clone of the genomic sequence of cystatin was obtained. The genomic clone was ~2.8 kb in length, and when sequenced it was found to include the entire cystatin gene. Comparison to the published cDNA sequence of the *A. viteae* cystatin (also termed as Av17) revealed that the genomic sequence also comprised 702 bp of the upstream genomic sequence containing the potential promoter region. The genomic

sequence of cystatin was found to comprise of four exons of 166 bp, 158 bp, 51 bp and 97 bp, interspersed by three introns of sizes 222 bp, 158 bp and 1020 bp and followed by 191 bp of 3' untranslated sequence (Fig. 3.1). The coding sequence of cystatin has an AT content of 59.7 % and a GC content of 40.3 %.The 5' putative promoter region has a GC content of 45 %.

Several putative regulatory sequences were identified in the 702 bp upstream genomic sequence using PromoterInspector and MatInspector public software analysis. A putative TATA-box was located at position –296 from the start ATG. Consensus recognition sites for the transcription factors NF-Y and AP-1 were identified at positions -37 and, -353 and -583 respectively. An inverted CCAAT box was located at position -603 (Fig. 3.1). In the 3' UTR in addition to the polyadenylation signals, the sequence ATTTA was found 137 bp downstream from the stop codon. Several transcription factor binding sequences in the putative promoter region suggests that the promoter is functional.

Figure 3.1. **The nucleotide sequence of the *Acanthocheilonema viteae* cystatin gene**

The gene sequence included the 5' upstream genomic sequence, four exons interrupted by three introns and the 3' UTR. The putative TATA box and transcription factor binding sites (CCAAT box, two AP-1 binding sites (CCAAT) and one NF-Y binding site (TGAGTCA)) are boxed. The sequence in red font corresponds to the 5' upstream genomic sequence and 3' UTR. Exons are in capital letters. The start ATG and the stop TGA are italizised

A previous study determined that *A. viteae* has three chitinase genes but only one functional transcript, that of gene I (Tachu et al., 2006, submitted). Therefore, the putative promoter region of only gene I was considered for this study. The genomic clone of *A. viteae* chitinase gene I including 1.6 kb of the upstream genomic sequence was kindly provided by Dr. Babila Tachu. Several putative regulatory sequences could be identified in the upstream genomic sequence (Fig. 3.2) using the MatInspector public software analysis. Consensus sequences of the NF-Y box were located at positions -244 and -1131 upstream of the start ATG. Two GATA binding factor 1 sequences were found at positions -964 and -1444 and an inverted CCAAT-box was found at position -1398 in the upstream genomic sequence. Also, a STAT 5 binding sequence and a transcriptional repressor were found at positions -1204 and -242 respectively. The presence of regulatory sequences suggests that the promoter of chitinase is functional.

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-1624 CTTTGGTAAT TAAACCATAG CAATGATAGT AAAAACACAA TATTAAAACA AGAAACANGC
-1564 GGTGCAAAGA ATAAATCAAA ACACATAACC TCAACGGAAG AATAAGTAGA AAGAATGAAG
-1504 AGATGAATGA TTTGATGTCT TATATAGGCA AAGTATACGT GCATATCATA ACGCGGGAAA
      GATA-binding factor                                  iCCAAT box
-1444 ATAAGGAAAG ATATTGGAGG ATATAAGAGC AAAGGAGATC ATGTAGTACA ATAATTGGAG
-1384 GCAAAGATGG GAACGGATGC GAGTATAGAA TGGATCATTT GAATGTGTTA TTTAGTTTAG
-1324 ACAGGAACAT CGACATTTGA CACGTATTAA CATGCAGTAA TATTCATTAT TACAATTATG
-1264 TCTATGAATA AAATATATAT ATATATATAT ATATCTTTCG CGACAAAAGG AATTGCTTTT
          STAT-5
-1204 CTCTGAAATT TTAATCCAAA ACAAAAAAAT TAATAAAAAT TAATTCTTCT TTGGTACTTC
                              NF-Y
-1144 TCGCTTTTCA CGTATCTTCC AATTAGTGAA GCCATGAATG AAAACATTAC CAACGCAAAC
-1084 GGTAAGTTGT CCAGTAAAGT GACAATTTTG GTTGGATGAT ATAGGTGGTA TATGGAATTT
-1024 TCACACTCTC AGATAAATGA AAAACGTACT TTGTGTTATA CGTCACATTT TCTTTTCACA
      GATA-binding factor 1
 -964 TTATGATAAA AGACATAACA AAATTCTCGA CAAGTACGGT ATTATTTGTG TTTTTCTAAC
 -904 TTTTAGGCTG TTGTTAATTT TTCCAGCTTT CCAGCTATTT ACGTATATTT TTCCATCTTT
 -844 TTTGGCCATT TCTTGTGTTT TTTCAGCTTT CTCGTGTTTT TCTAACTCCT TGAATATTTT
 -784 TCGTATTTTC ACAGCTTTTT AGAGGAGATG GTTTACATTT TCCTAAAGAA CGGTACGTTT
 -724 GATAAAGTAC TTTATAAAAG GCCCGTTTAT GGCGTTAAAG ACTTCACTTT TCATTTTATA
 -664 TTTTGCTATC TTTCAACATT CCTCCCGATT ACTTTACAAA AATATCTTTT TTCTATTTTT
 -604 GCACATTTAT CTTTTTTTTT GAATTTCTTT CTTTTTTGCT ATTTTCTAGA TATTTCCAGA
 -544 TTTTTAGTTT TGTATGCAAT CTCTGAGAAT GTCTTGCAAT TTGCGCAAAA ATAGAAAAAG
      Transcriptional repressor
 -484 ATATTTCATC GATATTTCTT AATTAGTCAT TTACATTAGC TGCAGCATAA CTCATTGAAT
 -424 TCCATATTTA TCCCAAAGAT TAGTATATCA ACATTATATT AAACGTTGAT ATACTAATCT
 -364 CATTTGGAAC AAAATGATAT CAAAAACATG AAAAATTTGC TACTTATAGA AAATACTGTA
 -304 CATCTAAGCT TACTTCTACT TCTCAATTAA TTTTTTCTTG AATCCTTTGT ATGCACCGAC
            NF-Y
 -244 AGTTATTCTC ACCAATCATT TCAATACAAT AATCTCTTAT TACTCTGTAA ACTCAGCAAA
 -184 AAAAGTATTC CTAATCGCTG AGCTATGTGT AATTAGTTAC GGTAATTCCA TGCATGTATA
 -124 TTAACTGATT GTTTCGCTTA TTTCTTACAC ATACACTTAA TATCTTCCAT TAATGAAGGC
  -64 AAATATCAGT TAATCATAGT AATGAATTTT GAAATACAGA AGTGTACAAG TCCCATGAAG
   -4 TGACATGATG
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Figure 3.2 The upstream genomic sequence of *Acanthocheilonema viteae* chitinase gene

The putative transcription factor binding regions of GATA, STAT-5 and NF-Y are boxed. The start ATG is italicized.

The functionality of the filarial promoters was determined in mammalian COS7 cells. The upstream genomic sequence of 702 bp of *A. viteae* cystatin was cloned 5' to an EGFP reporter with a SV40-polyA into the mammalian expression vector pSL1180 and was used to transfect COS7 cells. The cells were observed 24 h after transfection with a fluorescence microscope. The putative promoter region of 702 bp was capable of promoting transient expression of the reporter, EGFP (enhanced green fluorescent protein), in mammalian cells (Fig. 3.3). EGFP fluorescence was detected in 5 % of the COS7 cells transfected with the cystatin promoter-EGFP with the same intensity as the positive control, pEGFP transfected cells. COS7 cells transfected with a promoter-less control plasmid did not show any EGFP fluorescence. The 702 bp upstream genomic sequence of cystatin was enough to drive the expression of GFP suggesting that it is functional in mammalian cells.

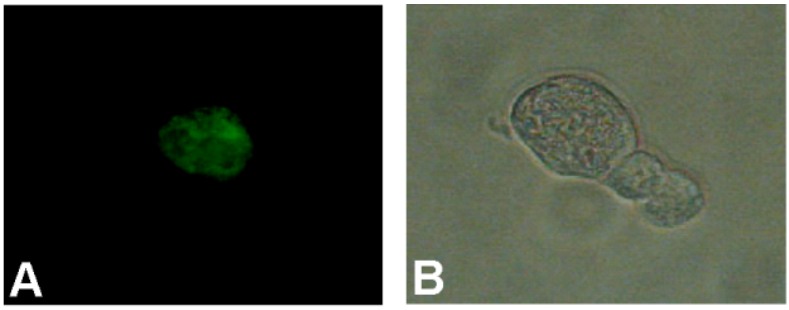


Figure 3.3 EGFP expression in COS7 cell under the control of *Acanthocheilonema viteae* cystatin promoter.

A: COS7 cell expressing GFP showing fluorescence in UV light

B: The corresponding cell in visible light

To determine if chitinase gene I promoter was functional the 1200 bp upstream genomic sequence was cloned 5' to the EGFP reporter with a SV40-polyA into the mammalian expression vector pSL1180. This construct was used to transfect COS7 cells. The putative promoter sequence of chitinase did not drive expression of EGFP in the COS7 cells. The positive control cells, transfected with pEGFP, expressed EGFP. This suggested that the upstream genomic sequence of chitinase used for the transfection is not complete to be functional and drive the reporter expression or that the promoter being nematode specific is not recognised by mammalian transcription factors.

3.1.2 Spatial expression of *A. viteae* cystatin in *C. elegans*

The putative promoter of cystatin was functional in mammalian cells whereas the 5' upstream region of chitinase was not. However, the presence or absence of expression driven by cystatin or chitinase promoters in mammalian cells does not necessarily imply the functionality in the filarial nematode. Therefore, the expression pattern of the filarial promoters was analysed in the free-living nematode *C. elegans*. The upstream genomic sequence of cystatin without or with the first exon of the gene was cloned upstream or as translational fusion with the GFP reporter in the plasmid pPD 95.77. These constructs were used to transform *C. elegans* worms. The promoter-less plasmid was used as a negative control. Transformation was carried out both by particle bombardment and microinjection.

3.1.2.1 Reporter gene activity in worms transiently transfected using particle bombardment

The temperature sensitive *pha-1* mutant of *C. elegans* was used in transgenesis experiments to facilitate selection of the transgenic worms. The transcription factor *pha-1* is required for the morphogenesis of the pharynx. In the pha-1 mutant worms the pharynx fails to undergo differentiation and the mutation interferes with the embryonic development at 25°C. This facilitates the selection of transgenic worms that are co-transfected with the marker plasmid (pBX) which rescues the transgenic worms to the wild type. The *C. elegans pha-1* worms were transfected by particle bombardment of 0.6 mg gold and 10 μg of plasmid DNA (construct and marker plasmid) with a pressure of 1500 psi. Nearly 60% of the worms were killed during the process due to the bombardment. No expression was observed in worms bombarded with constructs which had only the promoter sequence upstream to the reporter. However, expression was observed in worms bombarded with the translational construct (containing the promoter and the first exon of cystatin fused in frame to the reporter GFP). Expression of GFP was seen in all stages of the worms (L1, L2, L3, L4 and adults) depending on where the gold particles had lodged. Expression was mostly observed in the pharyngeal region with one of the gland cells showing fluorescence (Fig. 3.4). No expression was observed in the intestine or eggs even when gold particles had lodged there. Worms which showed the expression of GFP and also which had gold particles lodged in the gonads were transferred to fresh NGM plates to establish a transgenic line. This was

assuming that the gonad was bombarded with the constructs and marker plasmid which would rescue the pha-1 mutants and allow the progeny to survive at 25°C. Though the parent worms survived at 25°C, no transgenic progeny was established. Therefore, the expression was observed only in the parental worms. Although this showed that the *A. viteae* cystatin promoter is functional in *C. elegans,* it was transient expression and could not be maintained for more than one generation of *C. elegans*.

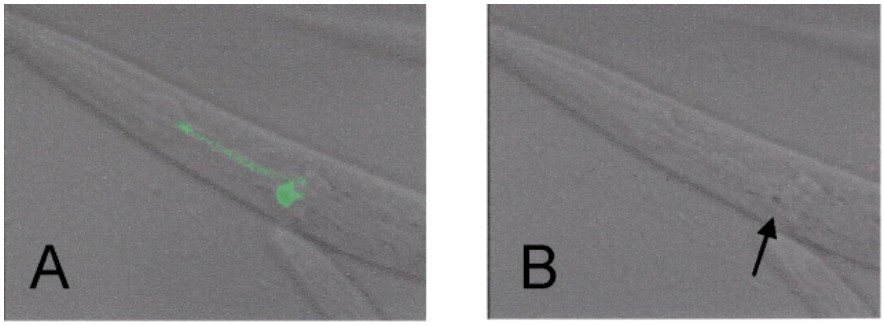


Figure 3.4 Transient expression of GFP driven by the *Acanthocheilonema viteae* cystatin promoter observed in the pharyngeal region of *Caenorhabditis elegans*.

A: Merged view of the worm in UV and visible light, with expression of GFP in the ventral g1 gland cell of the pharynx.

B: The worm in visible light with the gold particle lodged in the gland cell (black arrow).

3.1.2.2 Expression pattern in transgenic worms transformed by microinjection

A stable transformation was obtained by microinjection. Wild type (WT) *C. elegans* worms were transformed with a combination of the promoter construct and selectable marker plasmid (pRF4). The plasmid pRF4 contains a 4 kb *Eco*RI genomic DNA fragment of the *C. elegans* mutant collagen gene *rol-6(su1006)*, which causes the roller phenotype and was used as dominant selectable marker. After microinjection, individual worms were transferred on to NGM plates and allowed to recover for 3 days. By then the progeny had developed to the L2 or L3 stage. Only the transformed worms which showed both the roller phenotype and GFP expression were used to establish transgenic lines. Expression of GFP was observed in the transgenic line which was injected with the translational construct. In contrast, no expression was observed in the transgenic line which was injected with the construct that had only the promoter sequence upstream to the GFP. Expression of GFP was observed in the pharynx throughout the post-embryonic development but mostly in the L4 and adult stages. However, the roller phenotype was observed only from the

L2 stage onwards. Closer examination of the adult worms indicated that cells strongly expressing GFP were the gland cells of the pharynx and the rectum.

In general, the pharynx of *C. elegans* can be divided into 4 regions; the procorpus, the metacarpus (anterior bulb), the isthmus and the terminal bulb. The gland cells designate a set of 3 cells, 2 of which are synctial. The pair of g1 cells (dorsal g1 and right ventral g1) is fused. The g1 cells extend three cuticle-lined ducts anteriorly within the narrow pharyngeal nerve cords. Two of these ducts pass through the isthmus before emptying into the pharyngeal lumen near the metacorpus. The dorsal g1 duct travels much farther and empties near the anterior limit of the pharynx. Similarly, the g2 cells extend shorter ducts, which empty into the lumen of the terminal bulb. The g1 cells contain a lamellar cytoplasm and a few vesicles, while the g2 cells have a rather clear cytoplasm and more vesicles. The expression of GFP driven by the cystatin upstream genomic sequence was observed in the cytoplasm and the ducts of both the g1 and g2 gland cells (Fig. 3.5).

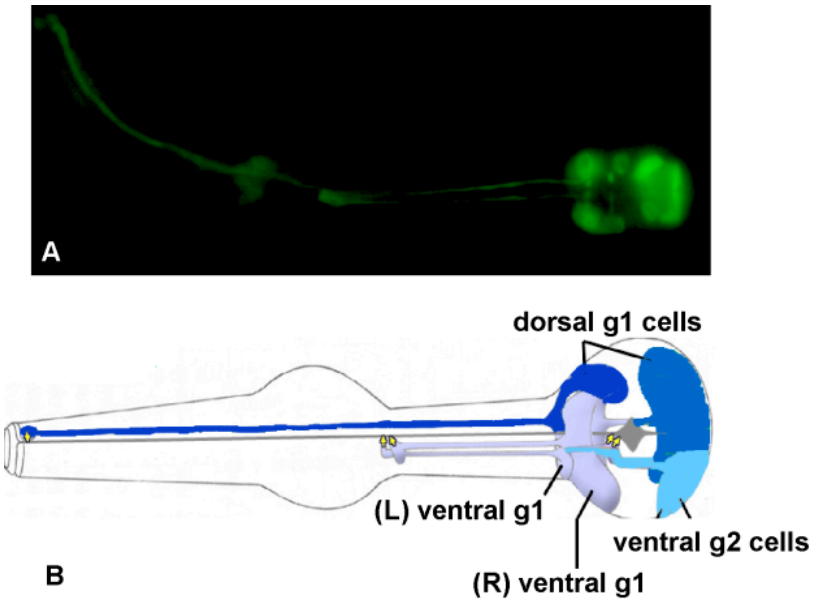


Figure 3.5. Expression of GFP driven by the cystatin promoter observed in the gland cells of the pharynx in transgenic *Caenorhabditis elegans*.

Expression was observed in the cytoplasm and ducts of the g1 and g2 gland cells of the pharynx of transgenic *C. elegans*. Source of B: www.wormatlas.org

Expression of GFP was also observed in the rectal gland of the transgenic line. The rectal gland consists of three large cells (recD, recVL and recVR) and is connected to the intestinal lumen just posterior to the rectal valve (Fig. 3.6). These three cells lie at the same level or just behind the rectal valve and their apical

specialization facing the lumen produces both microvilli and transitional epithelia in discrete patches.

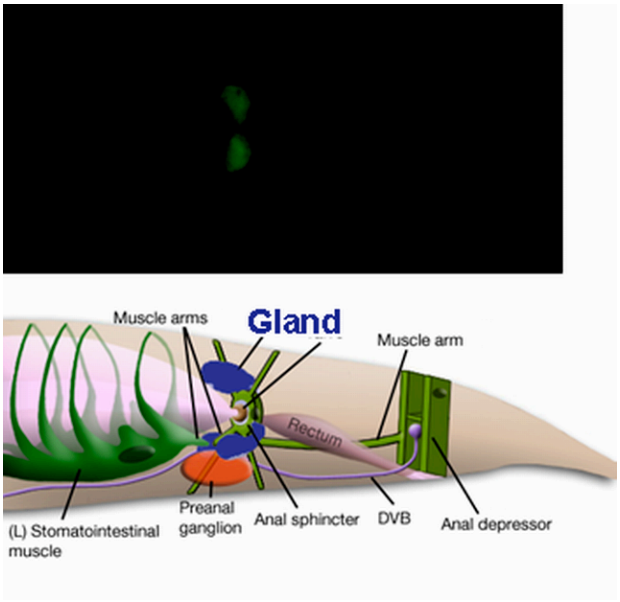


Figure 3.6. Expression of GFP driven by cystatin promoter observed in the 3 gland cells of the rectum of transgenic *Caenorhabditis elegans*.

The *A. viteae* cystatin promoter drove the expression of the reporter GFP in the three rectal gland cells (recD, recVL and recVR). Source of Fig. B: www.wormatlas.org

3.1.3 Functionality of A. viteae chitinase gene I promoter in C. elegans

C. elegans pha-1 mutant worms were particle bombarded with the construct containing the upstream genomic sequence of *A. viteae* chitinase gene I cloned 5' to the reporter GFP. The pBX plasmid was used as the marker plasmid. No expression of GFP was observed in the parental generation even though gold particles were lodged in the worms. Also, no transgenic line for only the marker, that allows the transgenic worms to survive at 25°C, was established. This suggests that the chitinase upstream genomic sequence of 1200 bp was not functional. This also implies that the chitinase promoter requires parasite specific cis and/or trans-regulatory elements to be functional even in the closely related *C. elegans.*

3.1.4 C. elegans as a system for expression of *A. viteae* cystatin

Earlier studies with *A. viteae* proteins, be it immunization or functional characterization, were done with *E. coli* expressed proteins. Therefore, the recombinant proteins did not have eukaryotic post-translational modifications and folding like their native form. It has been shown in various studies that protective

potential or functions may also be influenced by post-translational modifications of proteins. Therefore, to obtain filarial proteins most close to the native form, *C. elegans* was used as an expression system.

To obtain transgenic lines expressing *A. viteae* cystatin, *C. elegans* worms were injected with constructs containing the cDNA or genomic sequence of cystatin downstream to either an inducible or constitutive promoter. The temperature sensitive *pha-1* mutant of *C. elegans* was used for the microinjection experiments to facilitate selection of the transgenic worms expressing *A. viteae* cystatin. Transgenic worm lines, 103cAv173' (injected with p103Av17), 49cAv17 (injected with p49Av17c) and 49gAv17 (injected with p49Av17g) were established and maintained as discrete lines. The injected plasmids form large extra-chromosomal arrays which can silence the gene expression or induce a mosaic expression and/or are usually lost during future generations. In order to integrate the plasmid constructs into the genome, worms of the transgenic lines were exposed to gamma irradiation and screened for transgenesis. The F2 and subsequent generations of transgenic lines were used for further analyses. Single worm PCRs with the specific primers for cystatin to test for the presence of injected constructs showed that the worms (49cAv17, 103cAv173' and 49gAv17) were transgenic (Fig. 3.7). No product was amplified from the control *pha* -1 worms.

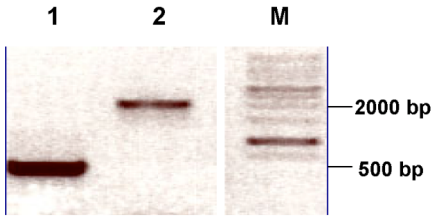


Figure 3.7 Single worm PCRs with transgenic *Caenorhabditis elegans*

Single worm PCR of lines 49cAv17 and103cAv173' with Cystatin primers amplified the cDNA (lane 1) and genomic sequence (lane 2) of cystatin, lane M shows the marker.

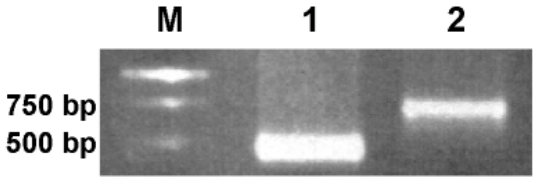
To obtain transgenic lines expressing *A. viteae* chitinase, *pha-1 C. elegans* worms were microinjected with construct containing the cDNA sequence of chitinase (p103Chi). No transgenic *C. elegans* lines expressing *A. viteae* chitinase could be made inspite of repeated attempts with different concentrations of construct.

3.1.4.1 Transcription of *A. viteae* cystatin in C. elegans

Expression of *A. viteae* cystatin at a pilot scale was tested on NGM agar plates. The plates containing the transgenic worm lines 49cAv17 and 49gAv17 were subjected to heat shock at temperatures varying through 30°C, 33°C, 35°C and 37°C. Worms treated with temperatures higher than 33°C died after an incubation period of 3 h. Hence, 33°C was considered as the optimum temperature for induction of expression of *A. viteae* cystatin in transgenic *C. elegans* lines 49cAv17 and 49gAv17. Large scale cultures of 200 ml of all transgenic lines were grown in S-medium with OP50 as a food source for three to four days at 25°C. The worms were harvested when worm numbers reached approximately 5 worms per μl and were cleaned by sucrose floatation to remove bacteria and debris. The lines 49cAv17 and 49gAv17 were re-suspended in S-medium and treated with a heat shock of 33°C for 3 h.

The transcription of *A. viteae* cystatin in *C. elegans* was shown by RT-PCR. A 475 bp product, consistent with the predicted size of the coding region was obtained by RT-PCR of the transgenic lines 49cAv17 and 103cAv17 containing the cDNA sequence of cystatin. In worms with the genomic sequence (49gAv17), transcripts of a larger size about 626 bp instead of the expected 475 bp were amplified (Fig. 3.8A). No amplification product was obtained from the control pha-1 worms. The products were sequenced. While the transcript in worms transformed with the cDNA had the right sequence, the larger transcript in worms transformed with genomic sequence was found to include a part of the first intron of the cystatin gene (Fig. 3.8B). Further analysis revealed that the first intron 5' splice donor site AG/gt was apparently read through and another AG/gt downstream in the first intron was recognised as the splice donor site. The splice donor site which was recognised by *C. elegans* had the consensus AG/gt but was different from the original donor site at the +5 position in the intron. The original 5' splice donor had a cytosine (c) while the second splice site recognised in *C. elegans* had a guanine (g) at the +5 position. Most *C. elegans* introns have a guanine at the +5 position in the intron. The other introns of *A. viteae* cystatin have a guanine at the +5 position in the introns like *C. elegans* introns and were, therefore, spliced out correctly (Table 3.1). Thus, the transcript of *A. viteae* cystatin transcribed in *C. elegans* had a size of 626 bp including 152 bp of the first intron instead of a transcript size of 475 bp, if splicing had occurred correctly.

A: RT-PCR



B: Schematic representation

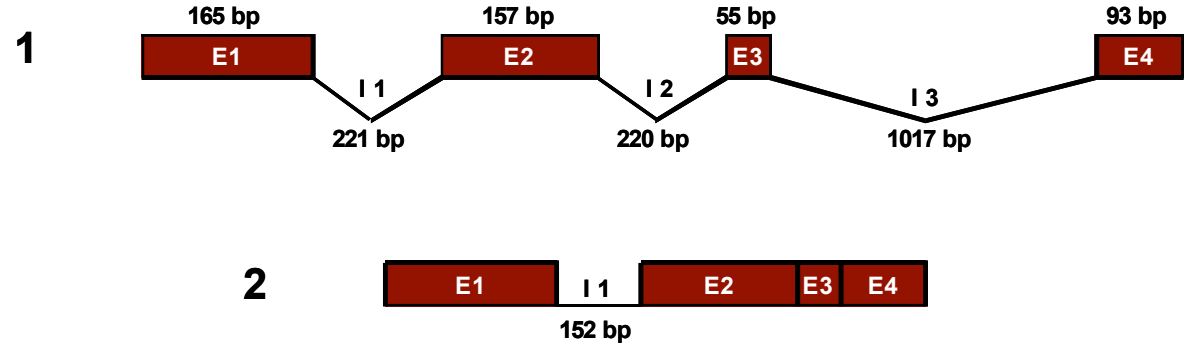


Figure 3.8 Transcripts of *Acanthocheilonema viteae* cystatin in transgenic *Caenorhabditis elegans* lines, 49cAv17 and 49gAv17.

A: RT-PCR

Lane 1: Transcript of *A. viteae* cystatin in the transgenic *C. elegans* line transformed with the cDNA sequence of cystatin(49cAv17); Lane 2: Transcript of cystatin in the transgenic line transformed with the genomic sequence of (49gAv17); M: Marker.

B: Schematic representation

The genomic sequence of cystatin of *A. viteae* (1) with the 4 exons (E) interspersed with 3 introns (I) and transcript of cystatin in *C. elegans* transgenic with the genomic sequence of cystatin (2).

Table 3.1: Sequences of splice donor and acceptor sites of cystatin in *A. viteae* and that recognised in *C. elegans.*

Intron	Size	5‘ Splice donor site in *A. viteae*	5‘ Splice donor site recognised in *C. elegans*	3‘ Splice acceptor site
1	221bp	ATACAG/gttacct	CGAAAG/gtttga	ttacag/GACCTG
2	220bp	AAAAAA/gtatgtt	AAAAAA/gtatgtt	tttcag/AGTTCA
3	1017bp	GATCAG/gtttgtt	GATCAG/gtttgtt	tttcag/ATTATC

3.1.4.2 Expression and purification of *A. viteae* cystatin from transgenic *C. elegans*

The expression of *A. viteae* cystatin in *C. elegans* was proven by Western blot. The total lysates of the transgenic worm lines, approximately 50 μg, were resolved by

SDS-PAGE and transferred onto nitrocellulose membranes, which were used for Western blot. When polyclonal antibodies raised against *A. viteae* cystatin (Av17) were used, a band of 17 KDa was observed in the transgenic lines 49cAv17 and 103cAv17 corresponding to the size of the *A. viteae* cystatin (Fig. 3.9). No expression of *A. viteae* cystatin was observed in the transgenic line, 49gAv17, and in the negative control of *pha-1* worms. This shows that the endogenous cystatin of *C. elegans* were not recognised by the serum. The western blots showed that the recombinant protein was expressed in *C. elegans*, though the levels were low.

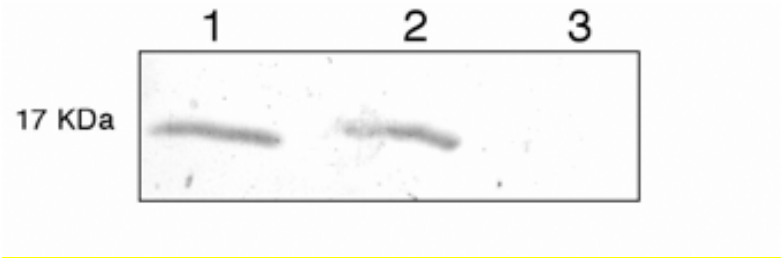


Figure 3.9 Western Blot with anti-cystatin antibodies shows the expression of *Acanthocheilonema viteae* cystatin in *Caenorhabditis elegans*.

A. viteae cystatin expression in transgenic lines 49cAv17 (lane 1) and 103cAv17 (lane 2), no expression was observed in 49gAv17 (lane 3).

A Ni-NTA column was used for the purification of the recombinant cystatin which is His tagged. The transgenic worms were lysed in lysis buffer by sonication and the supernatant was loaded onto Ni-NTA columns and eluted with a low pH elution buffer. Western blot with the different fractions showed that the protein was not purified since most of the protein did not bind to the Ni-NTA columns. Varying the buffer conditions with urea, to denature the proteins and hence increase the binding to the column, also did not lead to the purification of the recombinant protein. Using larger volumes of the transgenic *C. elegans* cultures also did not lead to the purification of cystatin.

3.1.4.3 Effect of expression of A. viteae cystatin on C. elegans

The transgenic line of worms, 103cAv17, expresses *A. viteae* cystatin under the control of a constitutive promoter *let-858*. Therefore, the protein is expressed in all somatic cells during the whole life cycle. It was observed that the NGM plates containing this transgenic line had fewer worms than plates with control *pha-1* worms or the transgenic lines 49cAv17 and 49gAv17. To quantify this, a single young adult hermaphrodite from the lines 49cAv17 and 103cAv17 each was transferred to

individual NGM plates. This was done in sextuplets. The worms were left in the NGM plates for 20 h to lay eggs after which the adults were removed from the plates. In the line 103cAv17 where cystatin is expressed constitutively, of 20 (±15.9) eggs only 5.2 (± 3.8) developed to adults. Whereas, in the transgenic line 49cAv17 where cystatin is induced only with a heat shock, of 24 (±16.4) eggs 19 (±10.2) developed into adults ($p <= 0.0129$). The reduction in the development in the line 103cAv17 was because 65 % of the eggs were arrested and did not develop further to the L1 stage (Fig. 3.10). This suggests that filarial cystatin expressed in *C. elegans* interferes with the moulting or that expression of a foreign gene is toxic for *C. elegans*.

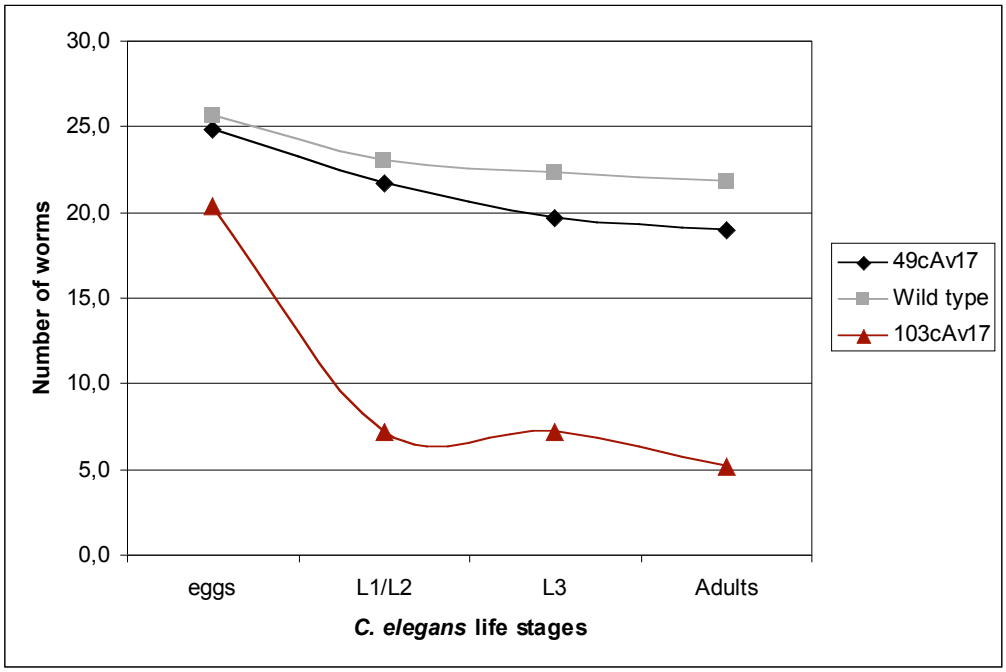


Figure 3.10 Effect of expression of *Acanthocheilonema viteae* cystatin on *Caenorhabditis elegans*.

The number of healthy larvae or worms of *C. elegans* transgenic lines expressing filarial cystatin either constitutively (103cAv17) or with induction (49cAv17) as compared to the progeny of wild type worms is plotted.

3.2 Functional characterization of cystatin and chitinase by RNAi

Cystatin and chitinase of *A. viteae* are potential intervention targets. While cystatin has been shown to be host immunomodulatory protein, its physiological functions in the parasite has not been studied. Similarly chitinase has been suggested to play a role in development of the parasite. However, no studies so far have determined its essential roles absolutely. In several organisms, including the free living nematode *C. elegans*, RNAi is being used in high throughput screens to

identify genes involved in particular processes [94, 95, 96, 97]. In *C. elegans*, gene silencing can be achieved by injection with dsRNA or dsRNA-expressing plasmids, by soaking in dsRNA solution or feeding worms on bacteria expressing dsRNA [77, 84, 85, 86]. In parasitic nematodes, successful RNAi has been reported in *Nippostrongylus brasiliensis, B. malayi, O. volvulus, Ascaris suum* and *Trichostrongylus colubriformis* [98, 99, 91, 92, 93, 100]. However, it cannot be assumed that RNAi will be effective in all nematode species, since differences in susceptibility to RNAi have been found within the *Caenorhabditis* clade [101]. In this study, we determined whether *A. viteae* is amenable to RNAi using the different methods of soaking and electroporation with either dsRNA or small interfering RNA (siRNA). Also, the potential endogenous functions of cystatin and chitinase of *A. viteae* were explored by knockdown studies.

3.2.1 Optimal conditions for RNAi in *A. viteae*

The physiological functions of genes, cystatin and chitinase, of *A. viteae* were analysed by knockdown of the genes, for the first time, in this study. Therefore, the best method to induce RNAi had to be determined and the experimental parameters had to be standardised. *A. viteae* were treated with double stranded RNA (dsRNA) or small interfering RNA (siRNA) of cystatin by either soaking or electroporation. *A. viteae* L3s isolated from the arthropod host were treated with different concentrations of dsRNA of full length sequence of cystatin (AvCys dsRNA) by soaking and by electroporation. The best effects were observed by the soaking method with 75.5 pmol / 100 μl of AvCys dsRNA and therefore this concentration was used for chitinase RNAi experiments as well.

Electroporation of L3s with AvCys dsRNA was done with varying the pulse voltages and lengths. The worms showed no effects, neither phenotypic nor decrease in transcripts, at different conditions and therefore electroporation with AvCys dsRNA was not used for further experiments. Three siRNA's (Cysi1, Cysi2 and Cysi3) of cystatin corresponding to different sequences which could potentially induce silencing were also used for RNAi at the L3 stage. The L3s were electroporated with varying concentrations of siRNAs, varying pulse voltage and length, as for the electroporation with AvCys dsRNA. No significant effects were observed using electroporation with any of the siRNAs. Soaking with siRNAs also did

not induce silencing or any phenotypic effects. The different conditions used for RNAi with cystatin are summarized in table 3.2.

Table 3.2 Standardisation of methods for RNAi of cystatin in A. viteae L3 worms

Method	**Phenotypic effects**
Soaking of L3 with AvCys dsRNA	Effect on moulting of L3 to L4
Soaking of L3 with siRNA's	No effect
Electroporation of L3 with AvCys dsRNA	No effect
Electroporation of L3 with siRNA's	No effect

Accordingly, further RNAi experiments were done by soaking with dsRNA. The specific effects of RNAi with cystatin and chitinase in detail are mentioned in the following sections. To observe if the various methods induced gene silencing, the transcripts were quantified by real-time PCR as explained in section 5.8.1.2.

The transcripts of cystatin in L3 *A. viteae* larvae treated with dsRNA and siRNAs were quantified by real-time PCR. The relative quantification was done as mentioned in section 5.13.3. The tropomyosin gene (muscle isoform) was used as the endogenous control in every sample since it is constitutively expressed and is a house keeping gene. Worms treated with Mal dsRNA were considered as the reference sample. The quantification was done by the comparative C_t method. The standard deviation was calculated for the triplicates of each sample. The graphical representation of the quantification of cystatin transcripts in L3s after RNAi by different methods is shown in figure 3.11. It was observed that there was a decrease in transcripts of cystatin only in larvae treated with AvCys dsRNA by the soaking method.

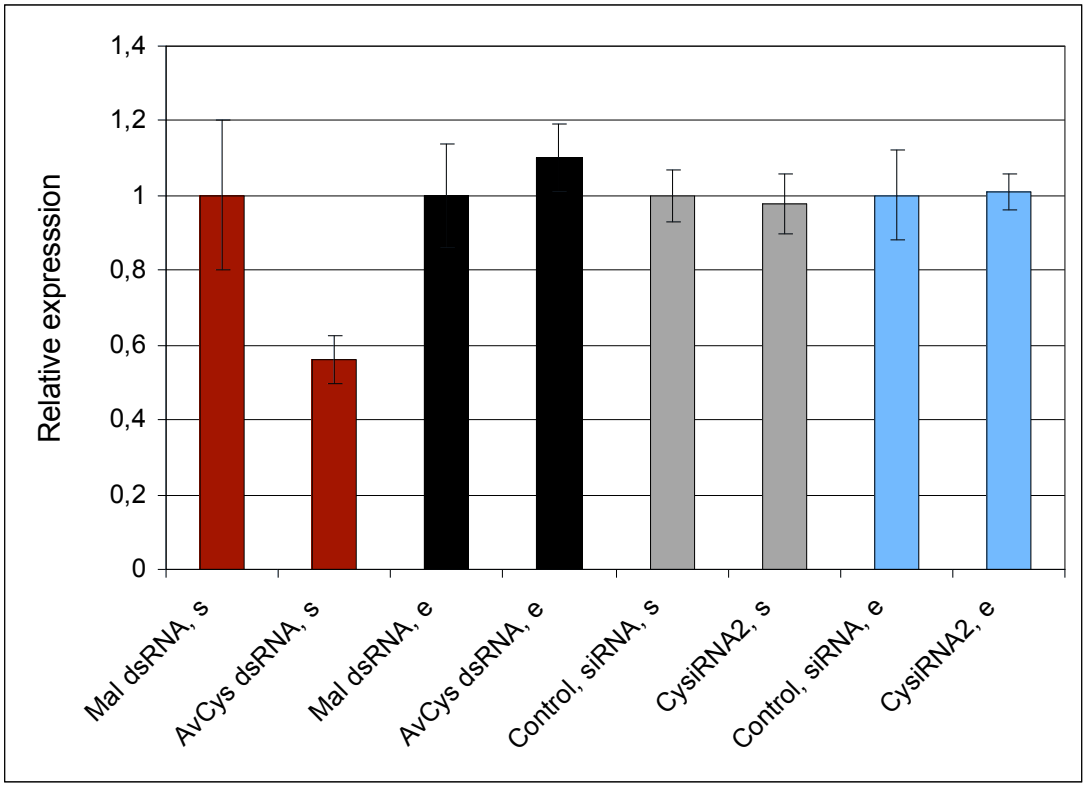


Figure 3.11 Relative quantification of cystatin transcripts in worms (L3s) treated to RNAi by different methods.

s: soaking, e: electroporation. The relative expression of cystatin in *A. viteae* L3s after RNAi by soaking and electroporation with either dsRNA or siRNA.

3.2.2 Role of cystatin during the developmental cycle of *A. viteae*

A. viteae cystatin has been well studied as an immunomodulator but it is suggested that it might also play a role in moulting. Therefore to determine the role of cystatin in the parasite, it was knocked down by RNA interference with dsRNA. The infective L3 stage and adults were treated with AvCys dsRNA and observed for the effects of knockdown of cystatin on moulting, infectivity (survival of parasite) and reproduction (microfilarial load) of *A. viteae.*

3.2.2.1 Knockdown of cystatin at the L3 stage delays moulting to L4

L3 larvae of *A. viteae* were soaked with AvCys dsRNA or negative control Mal dsRNA (75.5 pmol/100 μl) and observed for the effects on the phenotype. After 16 h incubation, the worms treated with AvCys dsRNA did not show any difference in viability or motility when compared to the worms treated with Mal dsRNA. Though, both groups (AvCys dsRNA and Mal dsRNA) seemed slightly lethargic as compared to the medium control where the worms were not treated with dsRNA. This is because dsRNA is acidic and at high concentrations adversely affects the worms.

In order to examine the consequences of cystatin gene silencing in L3, the worms treated with AvCys dsRNA and Mal dsRNA. They were used to infect jirds and the late L3s were isolated after 4 days. In general, the late L3s have a different phenotype from that of the early L3s with their gut starts to differentiate and therefore is visible clearly. The late L3s of the group AvCys dsRNA had no visible phenotypic difference from the control group Mal dsRNA with respect to their gut development. However, when we studied moulting, a clear difference was evident. The worms were considered as moulted when they shed their cuticle in the medium (Fig. 3.12). To quantify the number of worms that had moulted, the cuticles shed into the medium were counted.

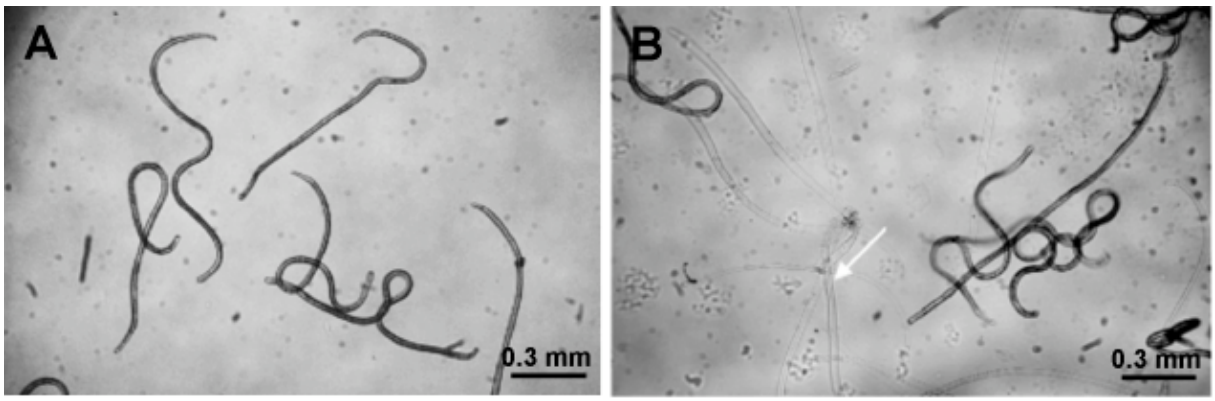


Figure 3.12 Moulting of the L3 larvae treated with AvCys dsRNA or Mal dsRNA (negative control)

The L3 worms moult to L4 and shed their cuticle in medium as observed at 48 h after recovering the late L3s from *Meriones*. A: Worms treated with AvCys dsRNA have not yet moulted. B: Worms treated with Mal dsRNA moulted to L4, the white arrow shows the shed cuticles.

The moulting rates of the L3 worms to the L4 stage are shown in figure 3.13. L3 larvae treated with AvCys dsRNA moulted significantly slower with 14% of the larvae moulted to the L4 after 48h. In contrast, 78.9% of the larvae treated with Mal dsRNA and 96.4% of the medium control larvae moulted to the L4 stage after 48 h (Fig. 3.13). However, by 122 h, 69.8% of the AvCys dsRNA treated L3s moulted to the L4 stage. This indicates that cystatin-knockdown delays moulting of the L3 to the L4 stage considerably. Moreover, 30 % of the L3 larvae did not moult to the L4 stage and therefore died.

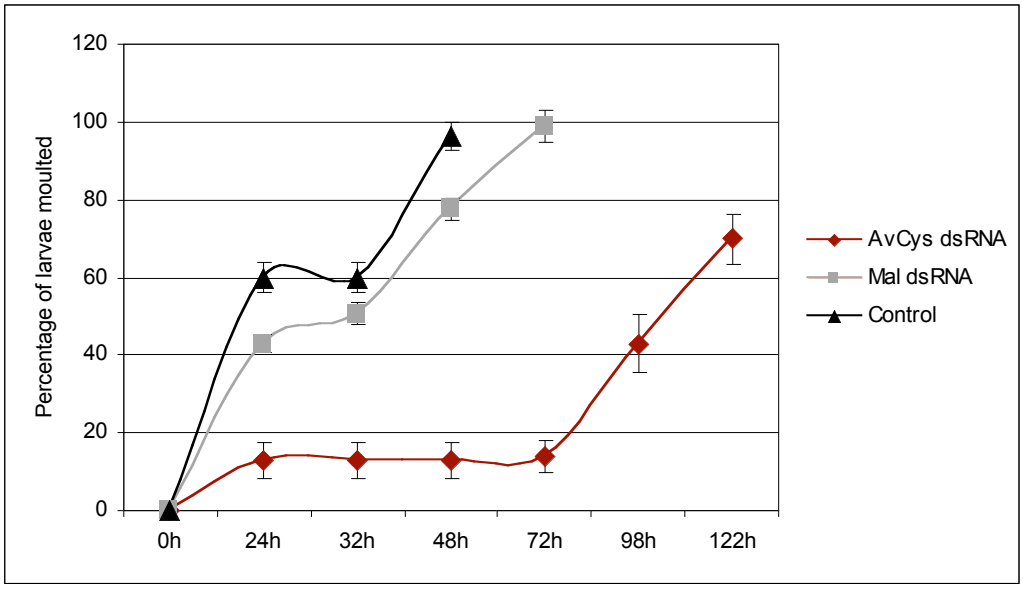


Figure 3.13. Moulting of the L3 larvae to the L4 after treatment with dsRNA.

The percentage of the larvae moulting to L4 in the groups of L3s treated with AvCys dsRNA (red line) are compared to that treated with Mal dsRNA (grey line) or medium control (black line).

3.2.2.2 Quantification of decrease in transcripts at L3 stage after RNAi with AvCys dsRNA

To confirm that inhibition of moulting of L3 to L4 stage was correlated to the silencing of cystatin at the L3 stage, transcripts of cystatin after treatment with dsRNA were quantified by real-time PCR. The tropomyosin gene was used as the endogenous control and worms treated with Mal dsRNA were considered as the reference sample. As compared to the Mal dsRNA larvae, the AvCys dsRNA group had a decrease of 47% (1.9 times less) of cystatin transcripts (Fig. 3.14). The decrease in transcript amounts support that moulting of L3 to the L4 is inhibited due to the knockdown of cystatin.

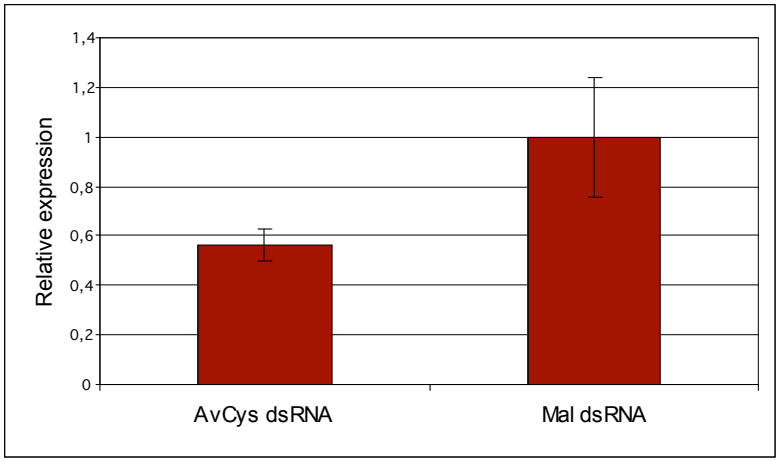


Figure 3.14 Quantification of cystatin transcripts by real-time PCR.

The transcripts of cystatin in L3 larvae of *Acanthocheilonema viteae* after RNAi by soaking with either AvCys dsRNA or Mal dsRNA are quantified by real-time PCR.

3.2.2.3 Effect on adult worms after knockdown of cystatin at the L3 stages

The effect of the knockdown of cystatin, at the L3 stage, on the infectivity and development of worms to the adult stages was analysed. The L3s were treated with either AvCys dsRNA or the control Mal dsRNA by soaking for 16 h and six *Meriones* were infected with 100 treated L3s each. The larvae were allowed to develop to adult worms and the worm burden was analysed after 11 weeks of infection. While the *Meriones* infected with the control Mal dsRNA treated worms had an average of 31 worms, the animals infected with AvCys dsRNA had only an average of 22 worms (Fig. 3.15). However, the differences in the worm burdens were not statistically significant.

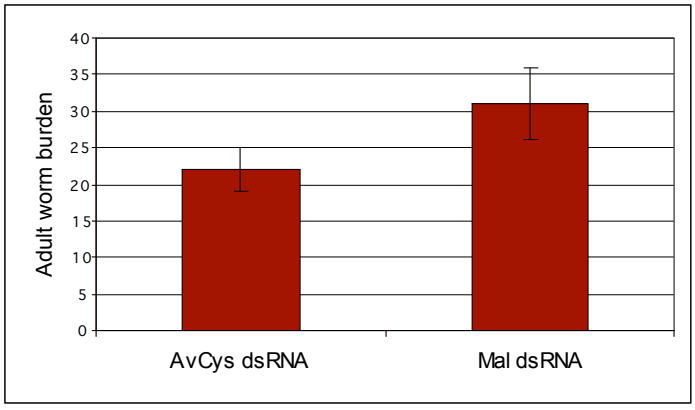


Figure 3.15 Average adult worm burdens of *Acanthocheilonema viteae*, in infected *Meriones*, treated at the L3 stage with AvCys dsRNA or Mal dsRNA.

Meriones infected with larvae treated with AvCys dsRNA had an average of 22 worms and *Meriones* infected with larvae treated with Mal dsRNA had an averagae of 31 worms.

This signifies a 30 % reduction in the development of L3 to the adult stage in larvae treated with AvCys dsRNA as compared to the Mal dsRNA treated larvae. The length and any defects in the morphology of the adult worms were also analysed. However, no visible phenotype differences were observed between the adult worms recovered from the AvCys dsRNA and Mal dsRNA groups. Cystatin of *A. viteae* is suggested to play a role in the development of microfilariae since immunostaining localizes cystatin to the uterus and in developing microfilariae (personal communication, Hartmann et al.). However, no differences were observed in the blood microfilarial count in *Meriones* infected with AvCys dsRNA or Mal dsRNA treated L3s. This suggests that though knockdown of cystatin at the L3 stages delays moulting to the L4 stage, the moulted worms develop to normal adults. However, as

shown in a previous experiment (Fig. 3.13) where 30 % of the L3s treated with AvCys dsRNA died since they did not moult, a 30% reduction of adult worm burden was observed probably because they could not moult.

The recovered adult female and male worms were analysed for any decrease in transcripts of cystatin by real-time PCR. Mal dsRNA treated worms were the reference sample and the tropomyosin gene was used as the endogenous control. A slight difference was observed in female worms which were treated with AvCys dsRNA with 9% less cystatin transcripts than control Mal dsRNA treated female worms. Also, male worms from the group AvCys dsRNA had 12 % decrease in transcripts of cystatin as compared to male worms from the Mal dsRNA treated larvae (3.16). However, the differences were not statistically significant.

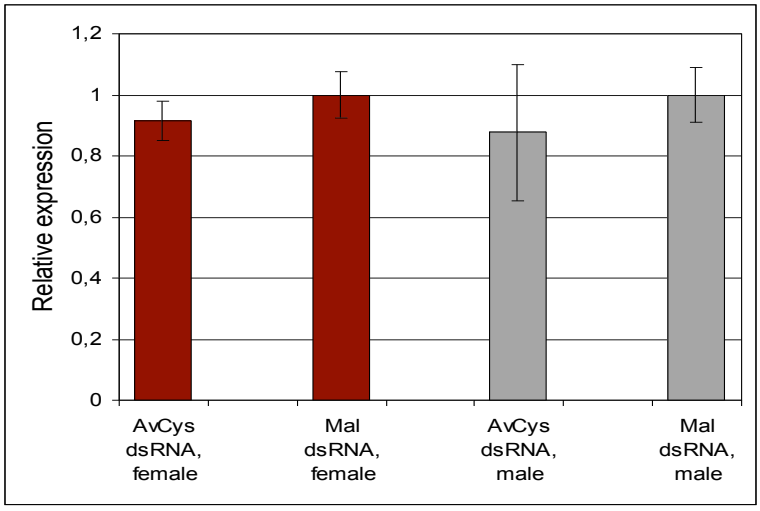


Figure 3.16 Relative quantification of cystatin transcripts in adult male and female *Acanthocheilonema viteae* treated with either AvCys dsRNA or Mal dsRNA at the L3 stage.

Relative expression of cystatin in female (red bars) and male (grey bars) worms treated with AvCys dsRNA at the L3 stage as compared to adult worms treated with Mal dsRNA at the L3 stage.

The worms were also analysed by activity assay to determine any effect on the activity of cystatin in the adult stage after RNAi at the L3 stage. The soluble protein extracts from the adult female and male worms which were treated with AvCys dsRNA or Mal dsRNA at the L3 stage were analysed for cystatin inhibition activity against papain, a cysteine protease. No significant difference in inhibition of cystatin was observed in either female or male worms of AvCys dsRNA treated L3 as compared to the female or male worms of Mal dsRNA treated L3s (Fig. 3.17).

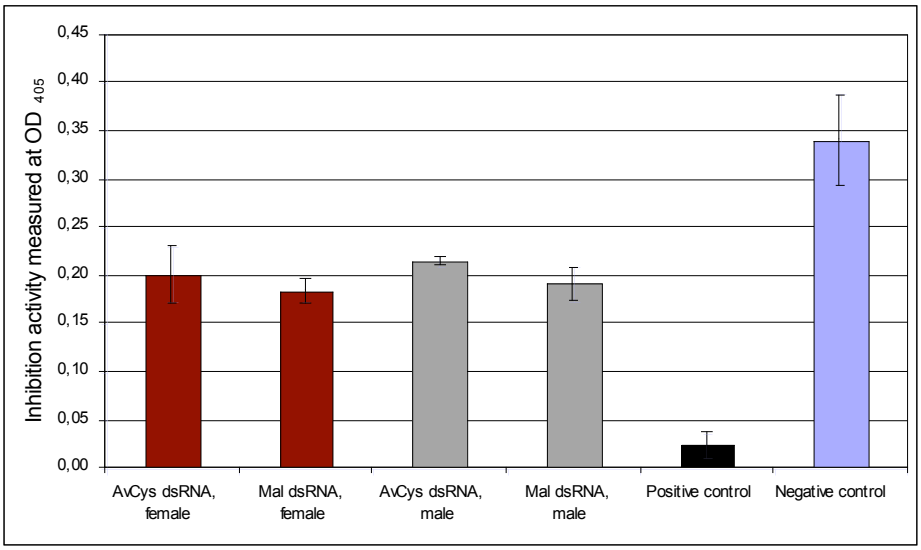


Figure 3.17 Inhibition activity of cystatin against papain in adult male and female worms treated with either AvCys dsRNA or Mal dsRNA at the L3 stage.

The inhibition activity of cystatin of adult female (red bars) and male worms (grey bars) treated with AvCys dsRNA or Mal dsRNA at the L3 stage. Recombinant *A. viteae* cystatin was used as the positive control (black bar) and only papain activity was used as the negative control (blue bar)

3.2.2.4 RNAi by soaking of adults in AvCys dsRNA

Adult *A. viteae* worms were soaked in AvCys dsRNA as mentioned in the methods section (5.12.1.3) to observe if they are amenable to RNAi. The adult female worms treated with AvCys dsRNA and Mal dsRNA at a concentration of 75.5 pmol / 100 µl seemed less motile as compared to the medium control worms. Of ten female worms in AvCys dsRNA and Mal dsRNA groups, 2 and 3 worms respectively died after incubation for 16 h. This was probably due to the high concentrations of dsRNA and also due to the low volumes, 300 µl, of iRPMI medium (without FCS). The rest of the worms recovered to normal motility like the control worms, after they were transferred to fresh medium. The worms were cultured for another 72 h to observe the effects of cystatin knockdown on the release and morphology of microfilariae. No decrease in the microfilariae release was observed in the group treated with AvCys dsRNA as compared to the medium control group and the Mal dsRNA group. This suggests that knockdown of cystatin at the adult stages had no effects either on the development and release of microfilariae or on the adult worms itself.

3.2.3 Essential role of chitinase during the developmental cycle of *A. viteae*

Filarial chitinases have been implicated to play a role on moulting and hatching. However, so far there have not been any functional characterizations of filarial chitinases by knockdown analyses. In *A. viteae,* of the three genes of chitinase, transcripts of only gene I of chitinase was observed. Therefore, to characterize the role of chitinase gene I in *A. viteae,* RNAi was done by the soaking method. DsRNA corresponding to the full length (1500 bp), which contains the glycosyl hydrolase domain, serine/threonine region and chitin-binding domain, of chitinase (AvChifl dsRNA) was used. In addition, the sequence between nucleotides 964 and 1464 (AvChi3' dsRNA) consisting of the serine/threonine region and chitin-binding domain was used. The adult worms and L3s were treated with AvChifl and AvChi3' dsRNA to determine the knockdown effects of chitinase gene I.

3.2.3.1 Knockdown of chitinase at the L3 stage inhibits moulting to L4

A. viteae L3 larvae were treated with AvChifl dsRNA, AvChi3' dsRNA and Mal dsRNA to validate any effects on the moulting to the L4 stage due to the knockdown of chitinase. After 16 h of incubation at 37°C, the L3s treated with AvChifl dsRNA and AvChi3' dsRNA did not show phenotypic differences as compared to the worms treated with Mal dsRNA or medium controls. The larvae were used to infect jirds and the late L3s were isolated 4 days post infection (p.i) and cultured for another 120 h. The moulting rate of the L3 to the L4 was quantified every 16 h. Significant number of L3 larvae treated with AvChifl dsRNA and AvChi3' dsRNA failed to moult to the L4 stage as compared to the Mal dsRNA or the medium control larvae. In AvChifl and AvChi3' dsRNA groups only 6 % and 7 % of worms respectively shed their L3 cuticles after 44 h. However, in the Mal dsRNA group 60 % of the larvae had shed their cuticles after 44 h (Fig. 3.18). This suggests that knockown of chitinase by both the dsRNAs considerably inhibits the moulting of L3 to the L4 stage implying that chitinase play an essential role in moulting of *A. viteae* larvae.

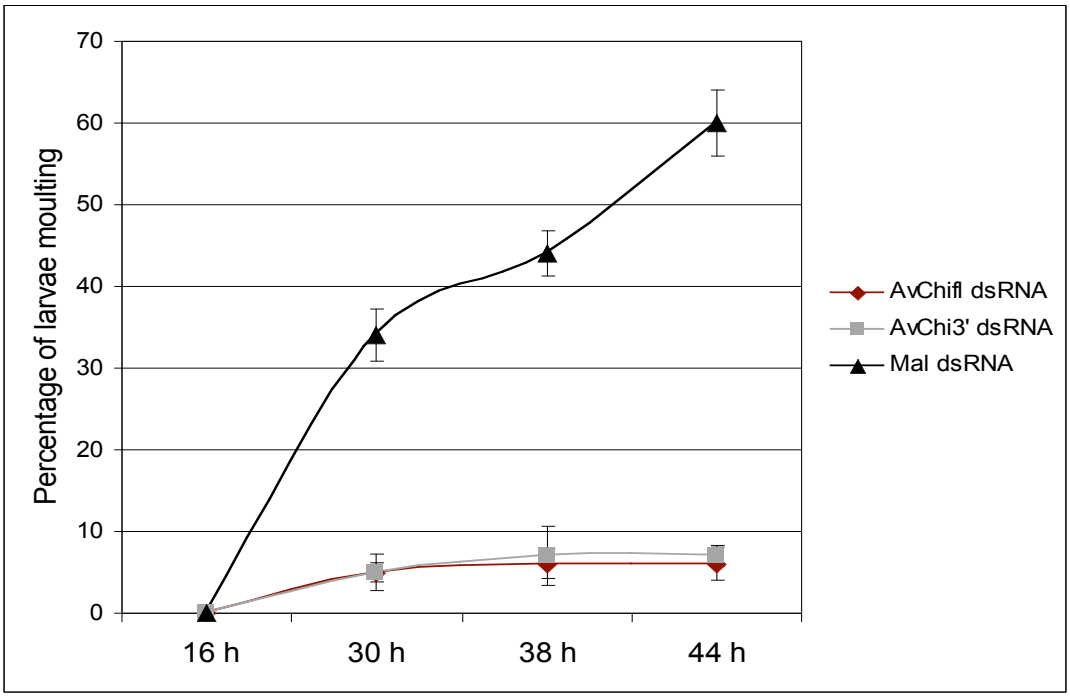


Figure 3.18 Moulting rates of L3 larvae to the L4 after treatment with dsRNA of chitinase.

The percentage L3 larvae moulting to L4 stage at different time points are plotted. L3 larvae were treated with AvChifl dsRNA (red line), AvChi3' dsRNA (grey line) or Mal dsRNA (black line).

3.2.3.2 Quantification of chitinase transcripts after RNAi at L3 stage

To test whether the phenotypic effect of inhibition of moulting was due to the knockdown of chitinase transcripts, the transcript numbers of chitinase in the L3 larvae were quantified by real-time PCR. Here also tropomyosin was used as the endogenous control and Mal dsRNA treated larvae were used as the reference group. The chitinase primer and probe were specific for gene I and therefore the readout was only for gene I. L3 larvae which were treated with AvChi3' dsRNA had 93% decrease or 14.3 times less of chitinase transcripts as compared to the Mal dsRNA group (Fig. 3.19A), confirming the silencing of chitinase. This in turn confirms that inhibition of moulting is indeed due to knockdown of chitinase. However, larvae treated with AvChifl dsRNA had about 43 times more transcripts of chitinase compared to the control (Fig. 3.19B) as determined by real-time PCR. To eliminate the possibility of template DNA, which was used for synthesis of AvChifl dsRNA, as a source of contamination, repeated DNase digestions were carried out after isolation of total RNA from the treated larvae. However, the real-time PCR results still indicated an upregulation of chitinase transcripts in larvae treated with AvChifl dsRNA.

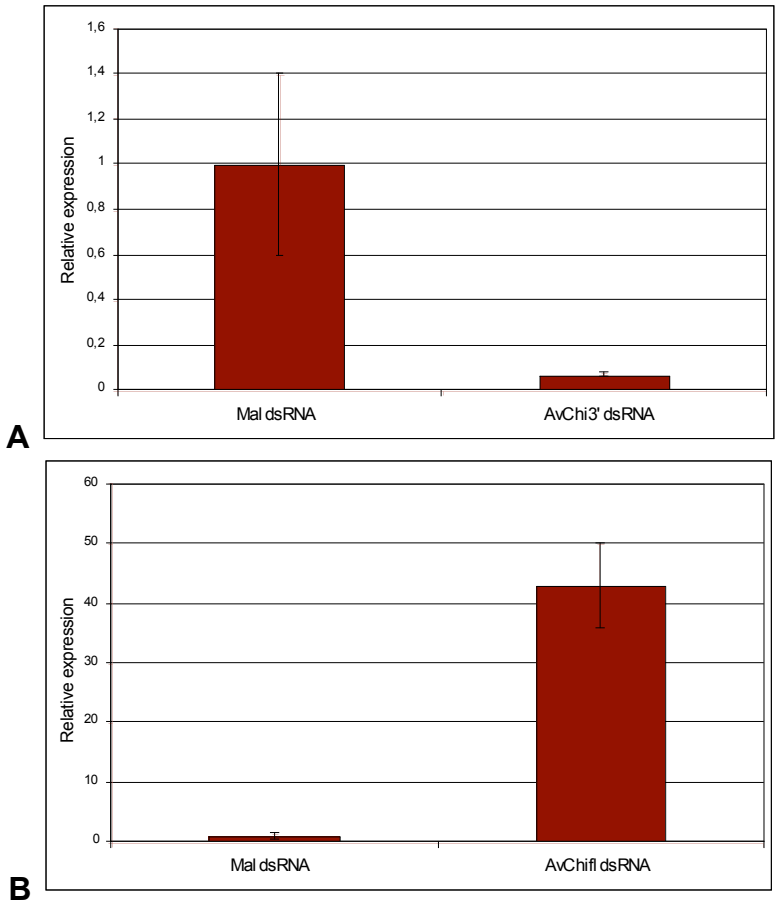


Figure 3.19 Quantification of chitinase transcripts in L3 larvae after RNAi.

A. Decrease in amount of chitinase transcripts, about 93%, in AvChi3' dsRNA treated larvae as compared to Mal dsRNA treated larvae.

B. Increase in amplicon amounts of chitinase, 43 times, in AvChifl dsRNA treated larvae as compared to Mal dsRNA treated larvae.

3.2.3.3 Knockdown of chitinase in adult A. viteae worms inhibits hatching of microfilariae

Filarial chitinase has been suggested to play role in hatching of the microfilariae since the egg sheath is made of chitin, the substrate of chitinase. Therefore, chitinase was knocked down in adult female worms to observe the effect on the development and hatching of microfilariae. Adult worms, both gravid females and males, were treated with AvChifl dsRNA and AvChi3' dsRNA as mentioned in methods section (5.12.1.3). The control worms were either treated with Mal dsRNA or no dsRNA in RPMI medium. After 16 h of incubation at 37°C, 80 % (8 of 10) of the female worms in the group treated with AvChifl dsRNA had died. A similar effect with a mortality rate of 60% (6 of 10) was observed in the female worms in the group treated with AvChi3' dsRNA. The female worms in the medium control and Mal dsRNA groups were still alive though lethargic. This suggests that the female worms

are affected by the knockdown of chitinase, indicating that chitinase plays an essential role in female worms. However, there was no difference in the morbidity and motility of male worms in all the groups. The worms were transferred to fresh medium with no dsRNA and cultured for another 120 h to observe the effects of knockdown of chitinase gene I on the release and morphology of microfilariae.

The release of microfilariae from the dsRNA treated female worms was quantified every 8-12 h for each worm. Worms treated with AvChifl dsRNA and AvChi3' dsRNA released a lower number of microfilariae into the medium as compared to the worms treated with Mal dsRNA or medium control worms. In the groups treated with AvChifl dsRNA and AvChi 3' dsRNA, the female worms released 50 % to 75 % less number of microfilariae at different time points than the worms treated with Mal dsRNA or with no dsRNA (Fig. 3.20). This indicates that knockdown of chitinase in adult female worms inhibits the release of microfilariae.

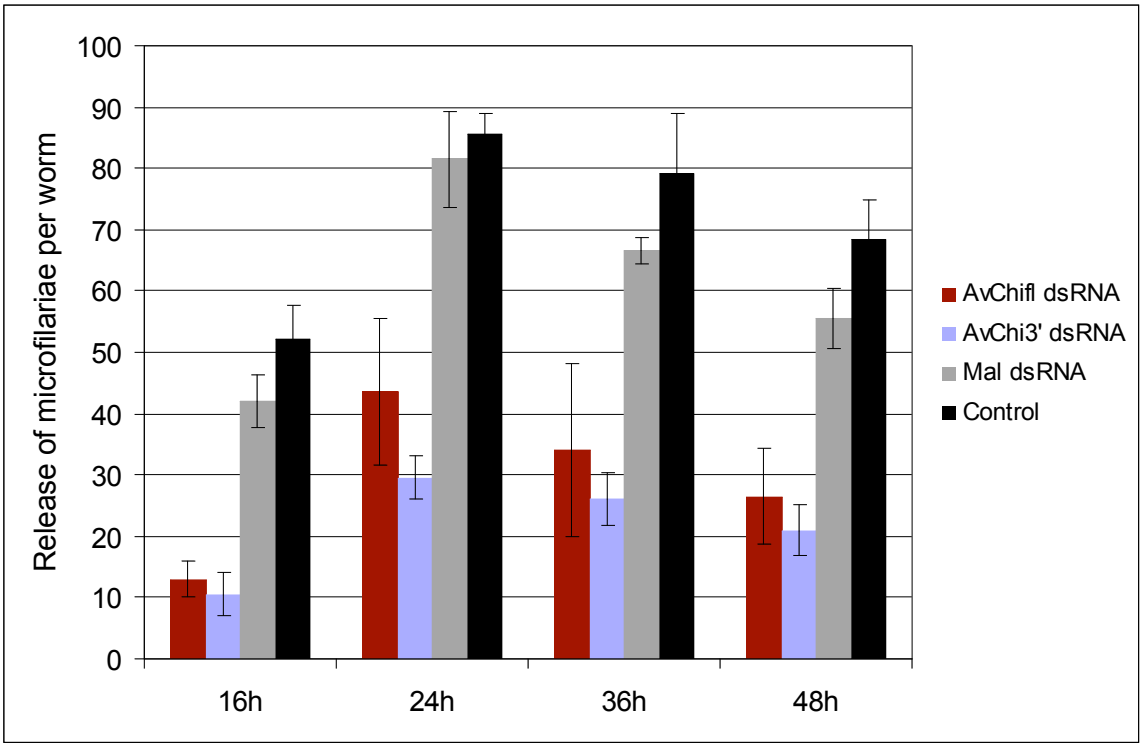


Figure 3.20 Release of microfilariae by *Acanthocheilonema viteae* adult female worms treated with AvChifl or AvChi3' dsRNA.

The release of microfilariae at different time points by adult female worms, in which chitinase was knocked by soaking with AvChifl or AvChi3' dsRNA, are plotted.

Normally, in *A. viteae*, the microfilariae develop within their egg shells in the uterus of the female worm. The mature microfilaria hatch within the uterus and are released into the host or medium. However in this study, the female worms treated

with AvChifl dsRNA and AvChi3' dsRNA, released unhatched microfilaria: The released microfilariae within their egg shells were mature. An average of 60% (± 15%) of the released microfilariae was unhatched in these two groups as compared to the control groups where all microfilariae had hatched before being released. This suggests that hatching of the microfilariae is inhibited due to the knockdown of chitinase (Fig. 3.21) and therefore resulting in the inability of the microfilariae to degrade the chitin in the egg shell.

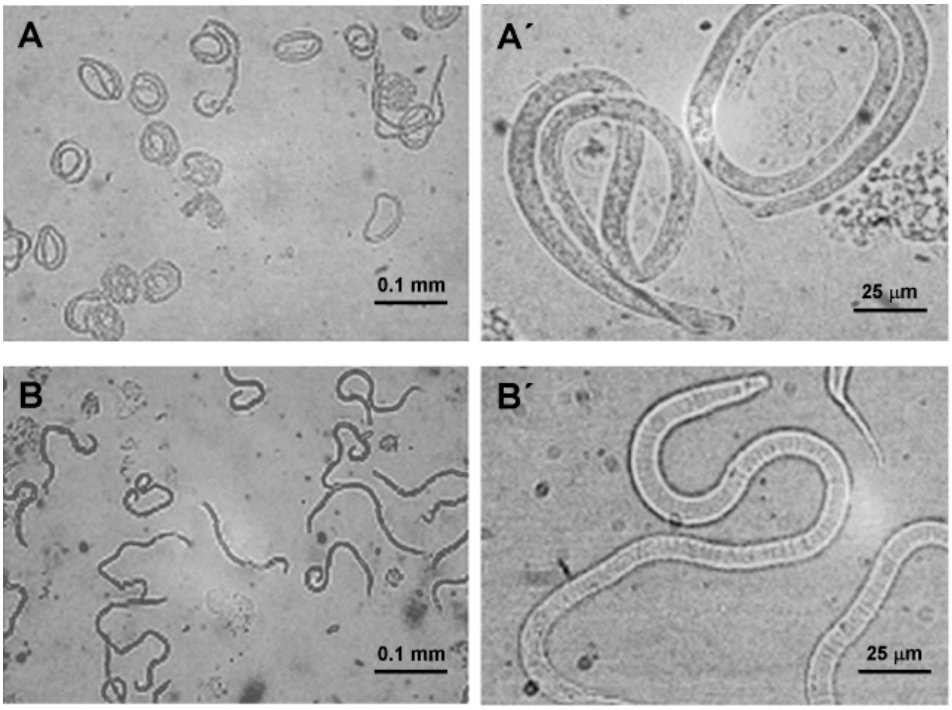


Figure 3.21 Inhibition of hatching of microfilariae after RNAi of chitinase.

A and A': Unhatched microfilariae released by adult worms treated with AvChifl or AvChi3' dsRNA.
B and B': Microfilariae released from female worms treated with Mal dsRNA.

3.2.3.4 Quantification of chitinase transcripts in adult worms treated to RNAi

To confirm that the phenotype observed in the releasing of microfilariae is indeed due to knockdown of chitinase gene I, the transcripts were quantified by real-time PCR. Worms treated with AvChifl dsRNA had 40% less transcripts and with AvChi3' dsRNA had 19% less transcripts of chitinase as compared to worms treated with control Mal dsRNA (Fig. 3.22). These results confirm the post-transcriptional silencing of chitinase gene by RNAi as observed by the phenotypic effects.

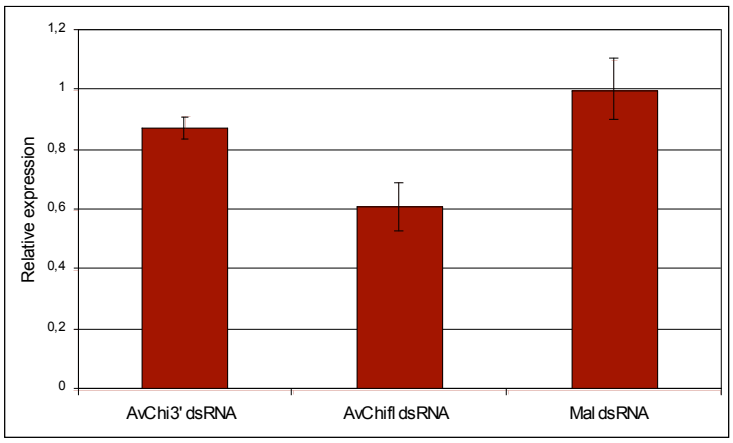


Figure 3.22 Quantification of chitinase transcripts in adult female worms after RNAi

A. viteae adult female worms were treated with AvChi3', AvChifl or Mal dsRNAs and the chitinase transcripts were quantified by real-time PCR.

3.3 Developmental expression of cystatin and chitinase in *A. viteae*

A. viteae like the other parasitic nematodes such as *O. volvulus, B. malayi, L. sigmodontis* have complex life cycles. By RNAi we determined that cystatin play an important role during L3 moulting and that chitinase is essential for the hatching of microfilariae and moulting of L3 larvae. These proteins were characterised at the adult or L3 larval stages since these stages are relatively robust to *in-vitro* conditions as compared to the other larval stages. However, this does not rule out that cystatin and chitinase may have essential functions in the other larval stages (L2, L4) as well. The timing of the gene expression at different stages would also give an indication to their functions during the developmental stages. In this study, real-time PCR with the different stages of *A. viteae* was done to provide an insight to the temporal regulation of cystatin and chitinase.

To quantify the regulation of cystatin and chitinase during the different stages of *A. viteae*, the transcripts were quantified by real-time PCR. The primers and probe for cystatin and chitinase were designed such that only transcripts and no genomic DNA were amplified (Table 5.5, section 5.13.1). Total RNA was isolated from the stages; eggs and intrauterine microfilariae, blood microfilariae, early L2, late L2, L3, L4 at 3 weeks post infection (p.i), L4 at 4 weeks p.i, early adults (6 weeks p.i), adult male and female. The worms sexually differentiate to the male or female worms 4 weeks p.i. However, the females have not yet started ovulation and therefore are free of oocytes and microfilariae. At 6 weeks p.i, the female worms have uteri, have started

ovulation and contain embryos at different stages of development. The cDNA was synthesized and used for the real-time PCR. Tropomyosin was used as the endogenous control since its expression is relatively constant during the different developmental stages.

3.3.1 Regulation of cystatin during the life cycle of A. viteae

The *A. viteae* cystatin was present throughout the developmental cycle; however its expression varied between stages (Fig. 3.23). Notably, the maximum expression of cystatin was found in the blood microfilarial stage and the minimum expression was observed in the adult male stage. The transcripts of cystatin during the different stages were therefore quantified relative to the adult male. As the uterine microfilariae (umf) hatched to the blood microfilariae (bmf) the amounts of cystatin transcript increased drastically with 6 times in umf to 100 times in bmf as compared to the adult male stage. However, the transcript amounts fell when the blood microfilariae developed to the L2 and L3 stages with nearly the same amount of cystatin transcripts in both the stages (14 times more as compared to the adult male worm). The cystatin transcript amounts decreased when the L3s moulted to the L4 stage which had about 2.5 times more transcripts than the adult male worms. As the L4 worms developed to female worms, the amount of cystatin transcripts increased with the young females having about 4.7 times more transcripts than the adult males. The expression of cystatin further increased to 6.7 times in mature adult female worms. Differently, as the L4s developed to males, the cystatin transcript amounts decreased with young males having about only 1.4 times more transcripts than adult male worms. The least amount of cystatin transcripts was found in adult male worms.

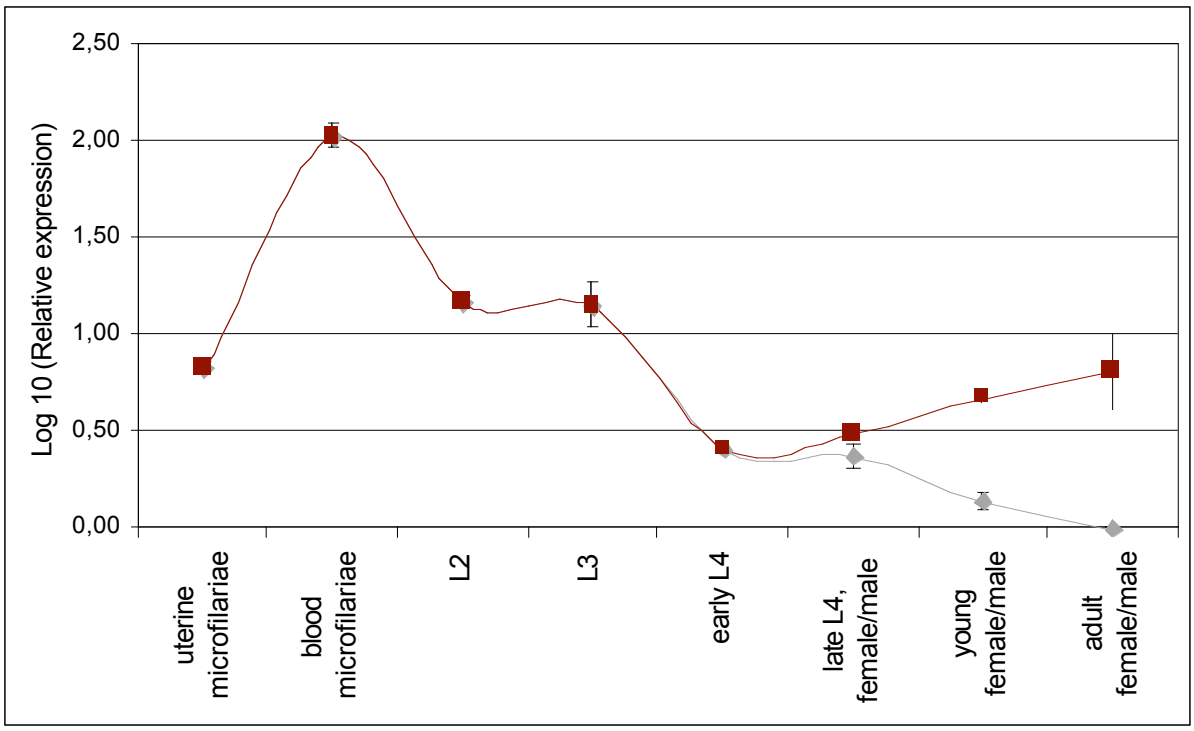


Figure 3.23 Regulation of cystatin during the life cycle of *Acanthocheilonema viteae*.

Logarithmic value of the relative quantification of cystatin as compared to the adult male is plotted against the different stages of *A. viteae*. Transcript amounts in females is shown in red line and in males shown in grey line.

3.3.2 Regulation of chitinase during the life cycle of A. viteae

Chitinase expression was found to be regulated during the various stages of the *A. viteae* life cycle. The maximum amount of chitinase transcripts was found in the infective L3 stage and the minimum in the late L4 male stage (Fig. 3.24). Intrauterine microfilariae and gravid adult females had the same amount of chitinase transcripts with about 15.5 times more transcripts than the late L4 male. The transcript amounts of chitinase decreased as the uterine microfilariae/eggs hatched to the blood microfilariae which had about 12.7 times more transcripts than the late L4 male. However, the transcript amounts increased as the blood microfilariae developed to the L2 (92 times more) and L3. The chitinase transcripts peaked at the L3 stage, with about 150 times more transcripts as compared to the late L4 male, and then decreased drastically at the early L4 stage with only six times more transcripts as compared to the late L4 male. The chitinase transcript amounts fell further as the early L4 stage sexually differentiated into the female or male. The least amount of chitinase transcripts was found in the late L4 male while the late L4 female had about 2.5 times more transcripts than the late L4 male. As the late L4 male and

female worms developed to the young adults, the transcripts of chitinase increased with 1.7 and 4.6 times more transcripts in male and female worms respectively. The gravid female adult worms had similar amount (15.3 times more than late L4 male) of transcripts of chitinase as the intrauterine microfilariae. However, the adult male had only half the amount of transcripts of chitinase as compared to the adult female (7 times more than the late L4 stage).

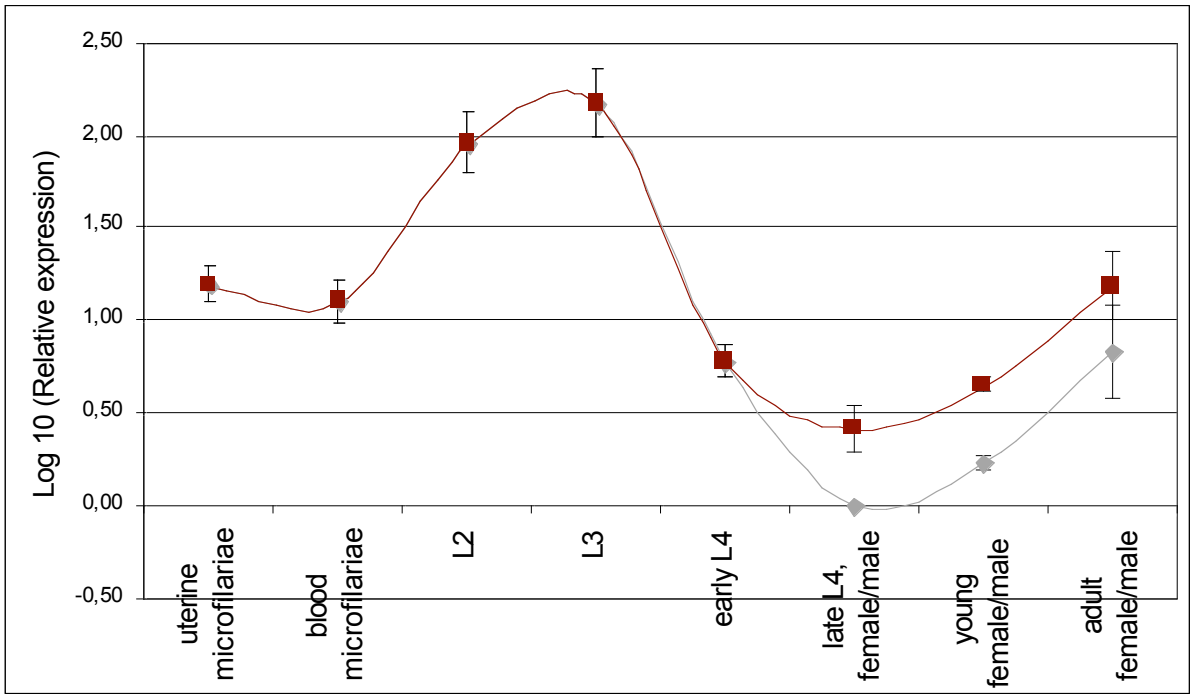


Figure 3.24 Regulation of chitinase during the life cycle of *Acanthocheilonema viteae.*

Logarithmic value of the relative quantification of chitinase as compared to the late L4 male is plotted against the different stages of *A. viteae*. Transcript amounts in females is shown in red line and in males shown in grey line.

Chitinase expression peaked at the L3 larval stage and RNAi experiments at this stage inhibited moulting to the L4. However, RNAi of chitinase at the adult stage also inhibited the hatching of microfilariae which also has considerable amounts of chitinase transcripts. Adult female *A. viteae* worms have oocytes and microfilariae at different stages of development in their uterus and chitinase in uterine microfilariae depicted in Fig. 3.24 represents transcripts from all these stages combined. Hence, it is of interest to determine if oocytes express chitinase during their development to mature microfilariae. Therefore, the uterine content from adult female worms was immunostained with monoclonal antibodies against chitinase (Fig. 3.25).

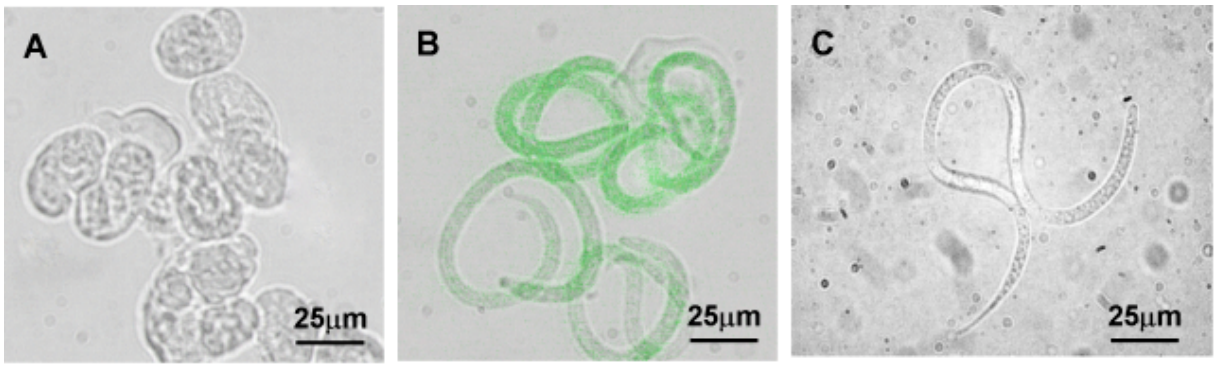


Figure 3.25 Uterine content of *Acanthocheilonema viteae* female worm immunostained for chitinase

Uterine content of *A. viteae* female worm which contains oocytes and microfilariae at different stages of development was immunostained using anti-Av-chitinase monoclonal antibodies as primary antibody and FITC-labelled anti-mouse as secondary antibody. A: Fertilized embryos at the comma stage, B: Mature microfilariae hatching from egg shell, C: Hatched microfilariae. The bars represent the scale.

Immunostaining of the uterine content showed that fertilized embryos do not have chitinase (Fig. 3.25A). Chitinase staining was observed in microfilariae which were about to hatch or was hatching (Fig. 3.25B) and not in microfilariae which had already hatched (Fig. 3.25C). This shows that chitinase expression in embryos is regulated coinciding with the hatching. This further confirms the RNAi results that determined that chitinase plays an essential role in the hatching of microfilariae.

3.4 Immunisation studies of *A. viteae* cystatin in *M. unguiculatus*

Cystatin which is an immunomudulator is an attractive vaccine candidate. Immunization studies with *O. volvulus* recombinant antigens suggest cystatin as a recombinant antigen vaccine [102]. The criterion for the selection of this antigen was its immunogenicity in infected individuals. *O. volvulus* cystatin was recognised by 65 - 90% of the infected individuals. *B. malayi* cystatin homologue, Bm-CPI2, present on the surface of adult and L3 worms has also been suggested to play a role in protective immunity [103]. Protein immunization is often restricted to major histocompatibility complex class 2-directed responses which does not always lead to a protective immunity. However, DNA vaccinations can elicit both cytolytic T cell and antibody responses, often mimicking the most effective immunological attributes of attenuated vaccines against pathogens. One of the objectives of this study was to evaluate DNA immunization of cystatin as an approach for inducing protective immunity in the rodent filarial model of *A. viteae.* Since a Th1 type of responses in

filarial infections such as *O. volvulus* is associated with the development of immunity in humans [104], intramuscular (i.m) mode of injection which induces antigen specific Th1 responses was used in this study. In addition, bacterial DNA contains CpG motifs [105, 106, 107] which induces non-specific Th1 effects [108], This is exacerbated by the amount of DNA required for effective i.m DNA immunisation. Vaccination with naked DNA by the intramuscular route relies on the ability of myocytes to engulf the plasmid [109] and some plasmid being endocytosed by antigen presenting cells (APC) infiltrating the site of injection or in the lymph nodes following its migration to the lymphatics. It has been proposed [110] that APCs are a preferred alternative to muscle cells as targets for DNA vaccine uptake and expression. Therefore, liposomes would be a suitable means of delivery of entrapped DNA to such cells. Moreover, liposomes would also protect [111] their DNA content from deoxyribonuclease attack.

In this study, the potential of cystatin as a vaccine against *A. viteae* infection was analyzed. Immunisation of Meriones were done with cystatin as protein, as DNA vaccine or as DNA vaccine encapsulated in liposomes. Immunisation experiments were done following the scheme on figure 3.26.

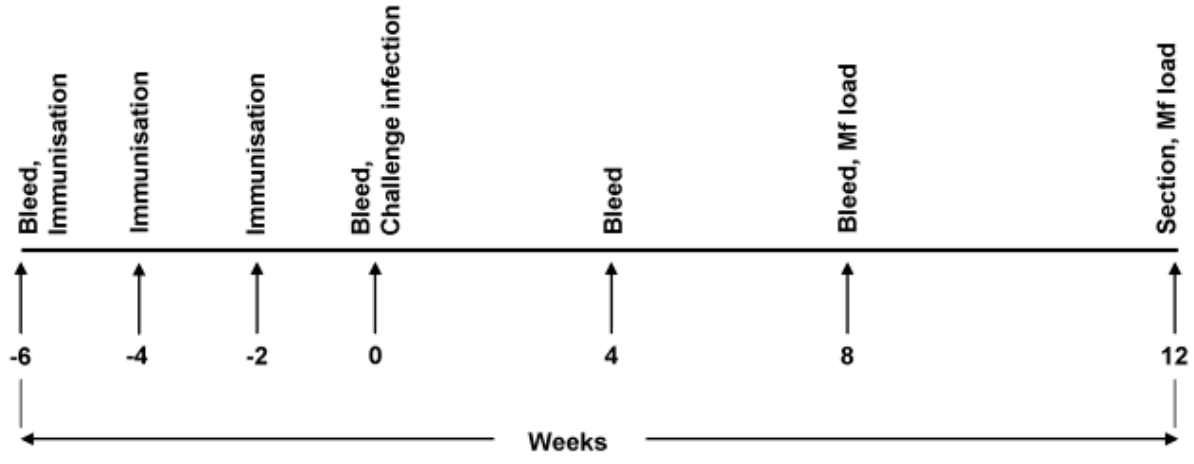


Figure 3.26 Immunization scheme of *Meriones unguiculatus* with *Acanthocheilonema viteae* cystatin

3.4.1 Immunisation studies with *A. viteae* cystatin expressed in *E. coli*

Recombinant cystatin of *A. viteae* was used in immunization of *Meriones* with STP or Alum as adjuvant. During the course of the experiment, one animal from the

Cystatin/Alum group, two animals from the Cystatin/STP group, three animals from the STP control group and three animals from the PBS control group died. Vaccination of *Meriones* with cystatin with either Alum or STP as adjuvant did not lead to a significant overall reduction in worm burdens as compared to the respective control groups (Fig 3.26). Also, no significant reduction in microfilariae numbers were observed in either Cystatin/Alum or Cystatin/STP groups (Table 3.3).

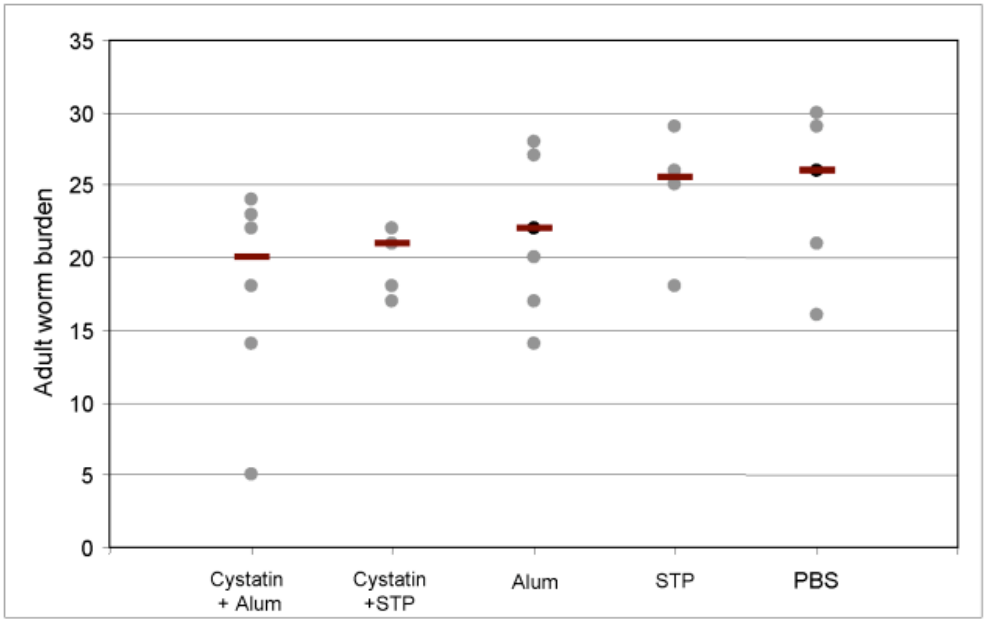


Figure 3.27 Adult worm burden in *Meriones unguiculatus* immunized with the *Acanthocheilonema viteae* cystatin as protein either with Alum or STP as adjuvant.

The grey circles denote individual immunized *Meriones* and the red lines denote the median of each group.

Table 3.3 Summary of biometric data from immunization studies with *A. viteae* cystatin.

	Cystatin/ Alum	Cystatin/STP	Alum	STP	PBS
Worms recovered	17.7 ± 7.2	20.2 ± 2.6	20.6 ± 3.6	24.5 ± 4.7	24.9 ± 4.8
Female worms	11.6 ± 5.3	12 ± 3.3	13.6 ± 3.5	14.25 ± 2.8	13.4 ± 3.5
Male worms	6 ± 3.5	8.5 ± 4.5	8.6 ± 4.2	10.3 ± 2.8	11.4 ± 2.7
Protection	14 %	17.5 %	-	-	-
Mf density / µl blood	11.5 ± 5.75	14.8 ± 5.5	13.2 ± 5	11 ± 5.8	21.6 ± 17.5
Surviving animals	6/7	5/7	7/7	4/7	7/10

3.4.2 Cystatin as a DNA vaccine

Meriones were also immunised with cystatin as a DNA vaccine. The mammalian vector plasmid containing the cDNA sequence of *A. viteae* cystatin

(pcDAv17c) was analysed for expression of cystatin in mammalian cells. No expression was observed in mammalian COS7 cells in a western blot using anti-cystatin antibodies. However, a RT-PCR for cystatin with the total RNA from COS7 cells transfected with pcDAv17c showed that *A. viteae* cystatin was transcribed. Also, the cDNA sequence of cystatin was cloned so as to obtain a translational fusion with EGFP (pcDAv17EGFP). When this construct was used for transfection of COS7 cells, expression of *A. viteae* cystatin as fusion protein with EGFP was observed by western blot (Fig. 3.28). Both the constructs, pcDAv17c and pcDAv17EGFP, were used for the DNA immunisation. The vector pcDNA 3.1 was used as a control. Ten-week-old *Meriones* were given the immunizations intramuscular at the thighs with 50 μg of plasmid in 100 μl of PBS.

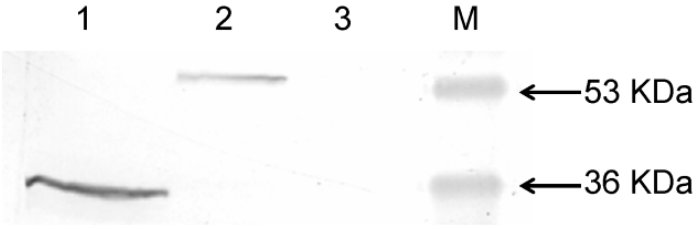


Fig 3.28 Expression of *Acanthocheilonema viteae* cystatin as EGFP fusion in mammalian COS7 cells

Western blot of total lysates of COS7 cells expressing GFP (lane 1), cystatin as GFP fusion (lane 2) and of untransfected cells (lane 3).

Intramuscular immunization of animals was done so as to transfect the muscle cells which would then express *A. viteae* cystatin driven by the CMV promoter of the vector. Animals immunized with pcDAv17EGFP in PBS showed a decrease (16.6 %) in worm numbers as compared to the control group. *Meriones* immunized with pcDAv17c/PBS showed a significant reduction (50.6 %) in worm burdens as compared to the control pcDNA 3.1 immunized animals in the first experiment. However, in the second experiment, only 28.5 % reduction in worm burdens was observed in *Meriones* immunized with pcDAv17c as compared to the control (Fig. 3.29). Also, no significant reduction in microfilariae numbers were observed in *Meriones* immunized with either pcDAv17c (Expts. 1 and 2) and pcDAv17cGFP (Expt. 1) as compared to the control pcDNA (Expts. 1 and 2) (Table 3.4).

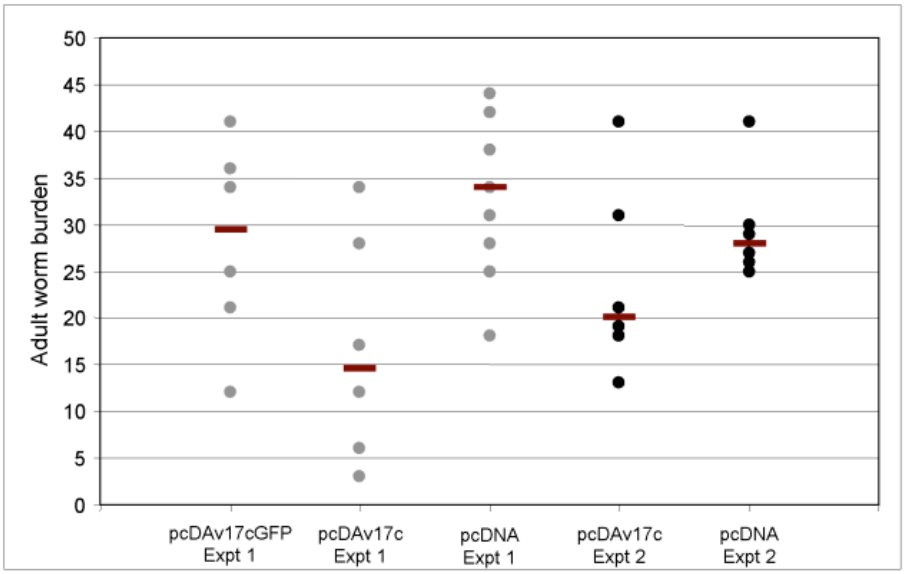


Figure 3.29 Adult worm burden in *Meriones unguiculatus* immunized with the *Acanthocheilonema viteae* cystatin as DNA vaccine in two different experiments.

The grey (Expt. 1) and black circles (Expt 2) denote individual immunized *Meriones* and the red lines denote the median of each group.

Table 3.4 Summary of biometric data from immunization studies with *A. viteae* cystatin as DNA vaccine

	pcDAv17cGFP Expt 1	**pcDAv17c Expt 1**	**pcDNA Expt 1**	**pcDAv17c Expt 2**	**pcDNA Expt 2**
Worms recovered	28.2 ± 9.9	16.7 ± 11.2	33.8 ± 8.6	20 ± 10.3	28 ± 5.8
Female worms	15.4 ± 4.8	8.5 ± 6.5	18.6 ± 4.5	12 ± 5.6	17 ± 3.5
Male worms	16.6 ± 6.5	5.8 ± 4.7	15.2 ± 4.3	9 ± 3.4	12 ± 2.7
Protection	16.5 %	50.6 %	-	28.5 %	-
Mf density / µl blood	25.3 ± 16.6	16.0 ± 8.5	30.1 ± 13.5	19 ± 5.7	21.8 ± 15.5
Surviving animals	5/6	6/6	8/11	6/7	6/10

Cystatin as a DNA vaccine against *A. viteae* infections in *Meriones* did not induce a protection which was reproducible. To improve the uptake of the DNA vaccine by the host antigen presenting cells, and therefore the protection, the plasmid DNA was encapsulated in liposomes. The plasmid DNA (pcDAv17c) was encapsulated in small unilamellar cationic liposomes (section 5.9). To investigate whether plasmid DNA was encapsulated, preparations were subjected to gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) at a concentration of 0.05% below the critical micelle concentration of the surfactant. Figure 3.30 shows that on gel electrophoresis of the preparation with SDS the plasmid DNA is displaced from the liposomes. This indicates that plasmid DNA was successfully encapsulated with the small unilamellar vesicles. The concentration of the plasmid DNA was

calculated by comparing the intensity of the plasmid DNA to the marker and accordingly was used for DNA-liposome immunizations. The encapsulated plasmid DNA (50 μg per animal) was used for immunisation with the same vaccination scheme as for plasmid DNA.

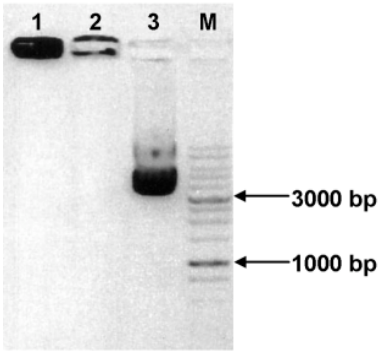


Figure 3.30 Gel electrophoresis of liposome encapsulated DNA

lane 1: liposome encapsulated DNA with no SDS, with 0.005% SDS (lane 2) and with 0.05% SDS (lane 3)

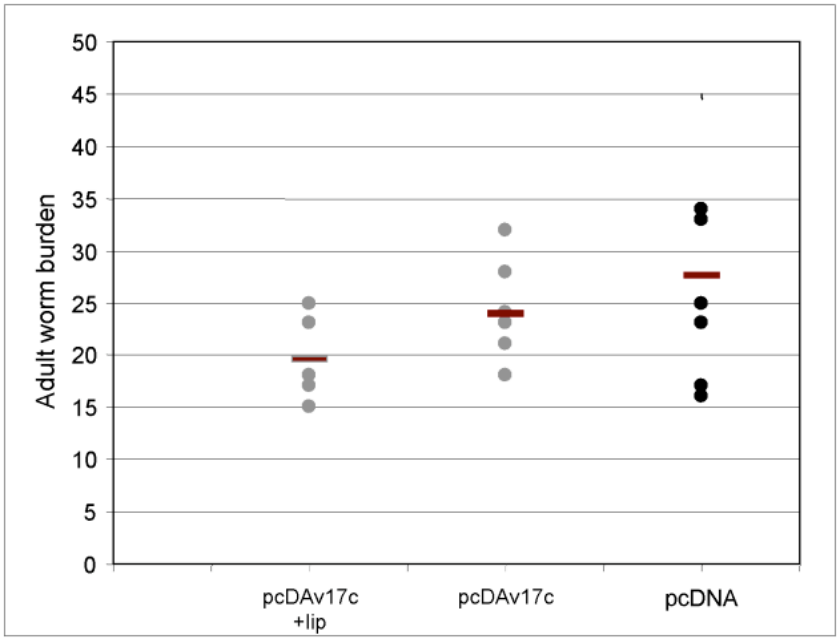


Figure 3.31 Adult worm burden in *Meriones unguiculatus* immunized with the *Acanthocheilonema viteae* cystatin as DNA vaccine encapsulated in liposomes

The grey (pcDAv17c+lip and pcDAv17c) and black (control-pcDNA) circles denote individual immunized *Meriones* and the red lines denote the median of each group.

Animals immunised with liposome encapsulated plasmid had 28.9 % reduction in worm burden as compared to the pcDNA control. This group had a higher

reduction in worm burdens than animal immunised with naked pcDAv17c, which had about 13.4 % reduction (Fig. 3.31). This suggests that liposome encapsulation of DNA vaccine increases the potential of the vaccine. However, the results were not statistically significant due to the high standard deviations and would have to be repeated. No significant differences were also observed in the microfilarial levels (Table 3.5).

Table 3.5 Summary of biometric data from immunization studies with *A. viteae* cystatin as DNA vaccine encapsulated in liposomes

	pcDAv17c + lip	pcDAv17c	pcDNA
Worms recovered	19.6 ± 4.2	23.9 ± 4.7	27.6 ± 10.39
Female worms	12 ± 4.8	12.0 ± 6.5	18.6 ± 4.5
Male worms	7.6 ± 3.8	5.8 ± 4.7	15.2 ± 4.3
Protection	28.9 %	13.4 %	-
Mf density / µl blood	19.3 ± 16.6	19.5 ± 8.5	17.3 ± 13.5
Surviving animals	5/7	7/7	7/9

4. DISCUSSION

4.1 C. elegans as a heterelogous system to study *A. viteae* gene promoters

One of the goals of this study was the functional characterization of the cystatin and chitinase (gene I) of *A. viteae.* Therefore, it was essential to determine the expression pattern of these proteins. This section describes the use of the free living nematode *C. elegans* as a system to examine the activity and specificity of the *A. viteae* cystatin and chitinase genes. Moreover, it demonstrates that it is possible to use *C. elegans* as a system for the expression of parasitic nematode proteins.

As a pre-requisite to study the expression pattern, the genomic organization of cystatin and the 5' upstream genomic sequences of cystatin and chitinase were analysed. The genomic sequence of cystatin was obtained from a λ dash genomic library. Only one gene of cystatin was obtained from *A. viteae*, although 2 isoforms of cystatin have been reported in other nematodes such as *C. elegans* [38], *B. malayi* [36] and *O. volvulus* [112]. The genomic sequence of cystatin contains three introns of sizes 222 bp, 158 bp and 1020 bp. The introns in the *A. viteae* cystatin gene are larger than 150 bp in length similar to the introns in the *C. elegans* cystatins (*cpi2a*

and cpi-1). However, the *C. elegans* cystatins have only two introns (Source: www.wormbase.org). The coding region of the *A. viteae* cystatin has an A+T content of 59.7 %, while the entire sequence of the gene including the introns has an A+T content of 65.6 %. However, the *C. elegans* cystatin 1 (*cpi-1*) has an A+T and G+C content of 50 % each. The high A+T content in *A. viteae* cystatin agrees with the known fact that filariids have some of the most AT-rich genomes [113, 114].

The 702 bp upstream genomic sequence of cystatin contains a TATA box, an inverted CCAAT box sequence and consensus recognition sites for the transcription factors AP-1, DSX (Drosophila sex determination factor) and NF-Y. Binding sites for the transcription factor AP-1 are found in numerous mammalian immunoregulatory and inflammatory genes [115]. In the promoter region of the mammalian salivary Cystatin S, the presence of an AP-1 binding site has been suggested to play a role in the regulation of expression [116]. Among parasites, AP-1 has been shown to be involved in the transcriptional regulation of *Schistosoma mansoni* calreticulin [117]. Differently, the promoter region of mammalian cystatin F is devoid of a typical TATA box element [118]. The presence of a CCAAT binding box in the promoter region may play a role in the regulation of expression of *A. viteae* cystatin since the CCAAT/Enhancer binding protein binding box found in the promoter region of the mammalian cystatin related epididymal spermatogenic (Cres) gene regulates its expression [119]. The upstream genomic sequences of *C. elegans* cystatins do not have transcription factor binding sites comparable to *A. viteae* cystatin. However, the *A. viteae* cystatin is similar to the mammalian cystatin F in its immunomodulatory properties and its preference for inhibition of cathepsin L protease rather than cathepsin B or S [120]. In the 3' UTR, in addition to the polyadenylation signals, the sequence ATTTA was found 137 bp downstream from the stop codon. It is known to regulate the stability of mRNA. This sequence element known as the AU-rich element (ARE) is found on many unstable mRNA's [121]. In this study, mammalian cells were transfected with the upstream genomic sequence of *A. viteae* cystatin driving the expression of EGFP. The putative promoter sequence was able to drive EGFP expression in mammalian COS7 cells, demonstrating that it is functional.

C. elegans was used as a heterologous system for studying the promoter activity of *A. viteae* genes. Defining genes of interest in parasitic nematodes is

difficult due to lack of appropriate knock-out approaches or suitable functional assays. Therefore, *C. elegans* is frequently used as a model system where genetic manipulation is relatively easy [64, 76, 66, 67, 122]. The expression pattern of *A. viteae* cystatin was analyzed in *C. elegans* transiently transfected by particle bombardment and in transgenic *C. elegans* lines obtained by microinjection of the translational construct. In worms bombarded with the translational construct, expression of GFP was observed in the gland cell of the parental worms. Though gold particles coated with the translational construct were lodged in other regions such as in the intestine and eggs, no expression of GFP was observed at those sites. This was probably because the promoter is not active in these regions. Earlier studies have successfully applied particle bombardment to obtain transgenic *C. elegans* lines [123, 124]. However, no transgenic line could be obtained in this study by particle bombardment. This could be since the amount of plasmid DNA used for particle bombardment was not optimal. Mello et al., [125] have shown that frequencies of transient expression and heritable transformation of *C. elegans* are dependent on the concentration of DNA.

Microinjection was used to obtain a transgenic line of *C. elegans* and the transformed worms were identified by the dominant selectable marker of the roller phenotype. The expression of GFP was observed in all larval stages and the adults of the transgenic *C. elegans*, showing that cystatin is temporally expressed as in the parasite, *A. viteae* [126]. The strongest expression was observed in the g1 and g2 gland cells of the pharynx and the rectal gland cells of transgenic *C. elegans*. Differently immunostaining by indirect immunofluorescence with antibodies raised against *A. viteae* cystatin localized cystatin to the hypodermis and in developing oocytes and embryos in sections of adult female worms (Hartmann et al., personal communication). Similarly, the expression of the *C. elegans* cystatin, Ce-CPI-2a, by immunostaining was observed in the gonadal sheath cells surrounding germ cells, oocytes and embryos [127]. Interestingly *cpi-2a* reporter construct (*cpi-2a* promoter driving reporter GFP) in transgenic *C. elegans* was observed to be expressed in many hypodermal and pharyngeal cells [128, 129]. Therefore, it can be assumed that the expression of cystatin in pharyngeal and rectal glands also occurs in *A. viteae* but as these nematodes cannot be transformed with reporter gene constructs as yet, an

analysis of expression in these glands would require extensive ultra-structural studies.

Alternatively, it is possible that the cystatin promoter sequence used is incomplete and requires additional regulatory elements to drive expression to the hypodermis. In the mammalian cystatin related spermatogenic (*Cres*) protein [119], the presence of gonadotrope-specific element (GSE) which interacts with SF-1 [130] was shown to be important for gonadotrope-specific expression. In their absence, the expression of *Cres* was different from that of the endogenous expression. Therefore, it can be assumed that the difference in the spatial expression, driven by the *A. viteae* cystatin promoter, between transgenic *C. elegans* and in *A. viteae* is due the lack of essential enhancer elements. Further studies that use promoter deletion analyses are required to establish whether smaller cystatin promoter fragments retain the ability to direct cell–specific expression of cystatin as in *A. viteae.* Transgenesis studies with *C. elegans* and *Strongyloides stercoralis* [131] have shown that transgenesis requires endogenous non-coding control elements in the 3'UTR. In the present study, the 3' UTR of cystatin was not used in the translational construct instead the *unc* 3'UTR of *C. elegans* present in the vector pPD95.77 was used. The inability of the *C. elegans* transcription machinery to correctly read filarial regulatory sequences in the upstream genomic sequence can also not be ruled out. Therefore, though *C. elegans* can recognise the filarial cystatin promoter, expression possibly does not display the same tissue-specific regulation that is observed in the homologous filarial environment.

However, the expression of the reporter gene in pharyngeal and rectal gland cells, i.e cells that release products to the exterior environment, is compatible with the earlier studies that have shown cystatin as an immunomodulator [38]. It is also congruent with the suggested function of cystatin in moulting. As these gland cells are stimulated synchronously with pharyngeal pumping activity, the question arises whether cystatin has a role in digestion. Generally, in *C. elegans* the gland cells have been proposed to store digestive enzymes, since digestion and absorption of nutrients take place in the gut. However, in filarial nematodes transcuticular transport is regarded as the main route for the absorption of nutrients [132]. This suggests that cystatin in filarial nematodes may not be involved in the regulation of digestive

proteases. Instead filarial cystatin may have evolved to become an immunomodulator that is permanently secreted by the parasites. This would be in line with the observation that molecules of the filarial worms have the capacity to induce the production of anti-inflammatory cytokines in host macrophages and change the phenotype of these cells, while *C. elegans* cystatins do not have these properties [38].

The genomic sequence of *A. viteae* chitinase gene I was obtained from λ-dash genomic library (Dr. Babila Tachu). The upstream genomic sequence, about 1600 bp, of chitinase gene I was analysed for transcription factor binding regions. The putative promoter region had two GATA binding factor sequences, 2 NF-Y boxes, STAT-5 binding sequence and an inverted CCAAT box. It also had a transcriptional repressor sequence. No TATA boxes or activator protein -1 binding sequence (AP-I) were found close to the start ATG. The AP-I site located close to the start ATG has been reported to play a role in the expression [133]. However, an absence of an AP-I sequence does not necessarily mean that the promoter is not functional [134]. Studies have suggested that the lack of an identifiable TATA box may be relatively common among the promoters of both parasitic and free-living nematodes. For example, TATA boxes have not been found in the upstream domain of the alt-1 gene of *B. malayi* [135], or in the OvSOD1, OvSOD2, OvGST-1a and OvGST-1b genes of the closely human filarial parasite *O. volvulus* [136].

Despite the presence of several transcription factor binding sites, the 1600 bp upstream genomic sequence of chitinase gene I did not drive the expression of the reporter, EGFP, in COS 7 cells. This suggests that the putative promoter sequence had transcription factor binding regions that were not recognised by the mammalian system. However, it cannot also be ruled out that for the functionality of the promoter, upstream or downstream regulatory sequences are required. *C. elegans* worms were transfected with the chitinase promoter::GFP reporter since the free living nematode is more closely related to the filarial nematode than mammals. However, no expression was observed in the transfected *C. elegans* worms, confirming that other regulatory sequences either upstream or downstream in the exons and introns are required for transcription. Immunostaining of the uterus content of adult female *A. viteae* showed that chitinase was expressed mostly in microfilariae just before and

after hatching. Expression was also observed in L3 larvae and in female adult worms but not in any other stages suggesting that chitinase in *A. viteae* is tightly coordinated during the life cycle. Therefore, the regulated expression of chitinase probably requires specific regulatory elements. In this study, since no expression was observed in transgenic *C. elegans* with the chitinase reporter construct it must have been due to the absence of parasite specific regulatory sequences. However reporter studies with the promoter of *C. elegans* chitinase (homologue C08B6.4) shows the expression in the hypodermal cells at the 3 fold and L1 stages of *C. elegans* [137]. The authors suggest that chitinase play a role in the cuticle formation in the L1 and in the initial moult to the L2. However, it cannot be assumed that the free-living and parasitic nematode chitinases show the same expression pattern. In the parasitic nematode *Ascaris suum*, chitinase belonging to the glycosyl hydrolase family 19 was shown to be secreted into the perivitelline fluid [137].

Filarial cystatin is a vaccine candidate [105] and we aimed to use *C. elegans* as an expression system. Many immunisation studies which compare *E. coli* expressed proteins and native proteins have shown that higher protection rates were observed when the protein was native [138]. Our own immunisation studies with *A. viteae* cystatin expressed in *E. coli* did not show significant reductions in worm burdens. *E. coli* derived proteins might be unsuitable because they lack critical epitopes owing to improper folding of proteins or absence of post-translational modifications. Therefore, *C. elegans* was validated as an alternative expression system to express *A. viteae* proteins in a form similar to native proteins. A possible advantage of using *C. elegans* as a host for expression of filarial candidate vaccine antigens compared to prokaryotic expression systems is that post-translational modifications, which might be relevant for inducing protective immune responses, would likely be conserved among the nematodes. Studies with native H11 protein from *Haemonchus contortus* have determined that the 90% protection obtained is due to conformation and/or glycosylation [139]. Similarly, glycan epitopes were suggested to be targets of protective antibody responses against intestinal nematodes [139, 140]. *A. viteae* cystatin is predicted to have one O-glycosylation and two phosphorylation sites. As well as glycosylation, folding of parasite proteins in their correct conformation may be essential for protection. Studies with *H. contortus* cathepsin L protease expressed in *C. elegans* have shown that it is functionally

active [76]. Furthermore, *C. elegans* can be grown in large numbers in a short duration obtaining sufficient amounts of protein would not be difficult.

The DNA and the amino acid sequence of *A. viteae* cystatin differ considerably from the *C. elegans* cystatins except at the evolutionarily conserved enzyme binding site [38]. The transgenic *C. elegans* lines, for *A. viteae* cystatin, obtained by microinjection were irradiated with gamma rays to integrate the large extra-chromosomal arrays into the genome. This was done since the maintenance of transgenic DNA as extra-chromosomal arrays may lead to gene silencing, mosaic expression of the protein and also loss of the trangenesis through many generations [125]. The stable lines after irradiation were maintained as discrete lines. The expression of *A .viteae* cystatin was detectable in the stable transgenic *C. elegans* lines (49Av17c and 103Av17c) by immunoblotting with anti-His antibodies and antibodies specific for *A. viteae* cystatin. However, the expression of the filarial protein was very low. Other studies have also suggested that it may be difficult to achieve significant over-expression of parasite proteins [122]. The recombinant protein could however not be purified over a Ni-NTA affinity column. This was probably because the His tag was not accessible due to steric hindrance by the protein. Furthermore, the transgenic *C. elegans* line (103Av17c) expressing cystatin constitutively was adversely affected with a decrease in the number of eggs hatching to the L1 and L2 stages. This suggests that probably the expression of protease inhibitor (cystatin) interfered with proteases involved in the differentiation or moulting of the worms. It is suggested that *C. elegans* is not ideal for expression of all parasite target antigens, particularly those not well conserved in *C. elegans* and which may be toxic when over-expressed [122]. Though the cystatins of *C. elegans* and *A. viteae* are conserved in the fact that they are inhibitors of cysteine proteases, they mostly differ in their functions. *A. viteae* cystatin has been shown to be an immunomodulator of the host responses whereas cystatin of the free living nematode, CPI-2a, does not have any immunomodulatory properties [38] and is involved in oogenesis and fertilization [127]. However, filarial and free living nematode cystatins have common non-essential functions such in moulting [127] as discussed later.

No expression of *A. viteae* cystatin was observed in the *C. elegans* line, 49gAv17, which was transformed with the genomic sequence of cystatin. However,

by RT-PCR it was found that *A. viteae* cystatin was transcribed. Surprisingly, the cystatin transcripts included a part of the first intron. A sequence 152 bp downstream in the first intron was recognised as the 5' splice donor site and has the consensus sequence of AG/gt. Apart from the 5' and 3' dinucleotide termini, the most conserved positions in the intron sequences are the five nucleotides from each end of the intron. The *A. viteae* cystatin transcript spliced in *C. elegans* has a guanine (g) at the +5 position (i.e., AG/gtttga) unlike the original donor splice site of the first intron of cystatin, which has a cytosine (i.e., AG/gttacc). Introns 2, 3 and 4 have a guanine at the +5 position. Most *C. elegans* introns [141] also have this conserved guanine at the +5 position of the donor site including those of the homologous cystatins. This suggests that the conserved guanine at the +5 position in the donor splice site might be necessary to be recognised as intron 5' splice donor site in *C. elegans*. The consensus sequences for junctions from parasitic nematodes, based on 55 introns, also show a guanine (g) at the +5 position in the first intron [143]. Most of the introns (70%) of *A. viteae*, chitinase, have the conserved G at the +5 position (Babila Tachu, personal communication), but there is not enough information on the organisation of the genes of this filarial species to allow a conclusion on the specificity of exon/intron recognition. However, in the closely related *O. volvulus* a guanine is found at the position +5 in 83 % of the introns [115]. The 3' splice intron acceptor sequence of uucag/A was recognised correctly and the other introns were spliced at the normal sites as in *A. viteae*. Earlier studies with the expression of *O. volvulus* and *H. contortus* proteins as GFP fusions in *C. elegans* showed that the introns were spliced out normally [73, 74]. Our results, probably for the first time, suggest that the intron splicing and recognition may differ among *C. elegans* and *A. viteae* at least with respect to cystatin. Owing to such differences, the expression of foreign genes in *C. elegans* might be more successful if cDNA sequences of genes are used, as compared to the use of genomic DNA.

4.2 Physiological functions of cystatin and chitinase in the lifecycle of *A. viteae*

The technique of RNA interference (RNAi) is a powerful means of suppressing the expression of specific genes [77] and offers great potential as a tool for studying gene function. In several organisms, RNAi is being used in high throughput screens to identify genes involved in particular processes [144, 145, 146, 147]. In this study cystatin and chitinase of *A. viteae* were targeted to analyse the knockdown effects

and thereby the functions of these proteins. The efficiency of RNAi depends on various factors such as the methods used, the gene targeted and the amenability of the parasite to RNAi. Therefore, RNAi with double stranded RNA (dsRNA) was standardised in *A. viteae* using the soaking method and the electroporation method. As an alternative to treating with dsRNA, we also tested whether RNAi could be induced in the presence of 22 bp synthetic small interfering RNAs (siRNA). It was observed that *A. viteae* is amenable to RNAi by soaking [84] demonstrating that the cellular components necessary for RNAi process are probably present in this species. In *C. elegans*, many of the essential proteins involved in this process have been identified, such as the translation initiation factor-related protein RDE-1, the RNAse III-related enzyme Dicer as well as the SID and RSD transporter proteins [148].

In this study the phenotypic effects and decrease in transcripts were observed only by the soaking method with dsRNA at a concentration of 75 pmol/100 μl while, electroporation killed the worms and lower voltage did not induce RNAi. Encapsulation of dsRNA with liposomes as reported other studies [149, 84] also did not induce RNAi (data not shown). This raises the question on the uptake of dsRNA by parasitic nematodes. Aboobaker and Blaxter [91] demonstrated in adult *B. malayi* worms that FITC-labelled dsRNA is localised to the hypodermis and digestive and uterine tracts, and the exact means of uptake in filarial nematodes, whether passive or active, is not known. Nematode amphids and phasmids, which are chemosensory organs, contain channels that open to the exterior through the cuticle and hypodermis [150]. It is probable that the uptake of dsRNA in L3s of *A. viteae* in this study may involve passage through the phasmidial and amphid ducts that are present on the surface of L3s. Lustigman et al [92] found that uptake of fluorescently labelled dsRNA was mostly in the esophagus and intestine of *O. volvulus* larvae, and also in the cuticular and hypodermal regions of the a few treated larvae, suggesting both ingestion and transcuticular pathways. Most of the earlier studies with parasitic nematodes such as *Heterodera glycines* [151], *Globodera pallida* [151], *B. malayi* [91] and *O. volvulus* [92] also observed RNAi with different genes by soaking with dsRNA.

4.2.1 Role of cystatin in the moulting of L3 to L4

Protease inhibitors play a variety of important biological roles by controlling endogenous and exogenous proteolytic activities. In parasitic nematodes they have been implicated in the parasite's survival within the host by inhibition of exogenous host proteases normally found in their preferred microenvironments [152, 153, 154, 155, 156], the inhibition of enzymes found in plasma or secreted from immune effector cells [157, 158, 159, 160], and the modulation of immune responses [160, 161, 112]. *A. viteae* cystatin is a well studied immunomodulator. However, its endogenous roles in the parasite have not being studied. In this study, we determined by RNAi that cystatin of *A. viteae* plays an important role in the moulting of the L3 larvae.

The cuticle is an extracellular hydro-skeleton that overlays the hypodermis of all nematodes. Most nematodes moult their cuticles four times during pre-adult development. Although being fairly inert and structurally robust, the cuticle is also permeable to small compounds and expands during growth periods between moults [162]. On soaking of *A. viteae* L3s with dsRNA of cystatin (AvCys dsRNA), the moulting to the L4 stage was observed to be delayed by 3 days as compared to L3s treated with the control Mal dsRNA. Moreover, 30% of the worms of AvCys dsRNA did not moult to the L4 stage which led to their death. These results confirm that cystatin in *A. viteae* plays a role in moulting possibly by controlling the maturation (through removal of inhibitory pro-regions) and regulating the activity of enzymes such as the cathepsins, which are cysteine proteases. RNAi studies have shown that knockdown of cysteine proteases of *O. volvulus* such as cathepsin L and cathepsin Z inhibits ecdysis of the L3 larvae [92]. The decrease in the level of cystatin transcripts were confirmed by real-time PCR. Worms treated with AvCys dsRNA had 43 % less transcripts of cystatin as compared to the control with the Mal dsRNA treated worms. This supports that the delay in moulting is due to the knockdown of cystatin.

The RNAi *in vivo* effects of cystatin knockdown were subsequently tested following introduction of the treated L3s into the mammalian hosts. *Meriones* infected with AvCys dsRNA treated L3 had 30 % decrease in the worm burden as compared to the control. This complements our *in vitro* results showing that 30 % of L3 died during moulting to the L4. However, the role of cystatin is *A. viteae* does not seem to be absolutely essential for moulting since the L3 larvae eventually moulted to the L4 stage, albeit with a delay of 2 days. The delay in the moulting of the L3 larvae did not

seem to affect their viability and infectivity. It is possible that despite reduced gene expression levels for part of the experimental period, and also for 5 - 8 days until ecdysis, disruption of cystatin protein levels was insufficient to interfere markedly with adult and microfilarial viability or development. Similar short term RNAi effects were observed in *H. contortus* with beta-tubulin [163]. It will be important to determine the longetivity of effects for knockdown of cystatin to analyse the other probable functions. Moreover, it is possible that not all worms were affected to the same extent. Such variation is consistent with results reported in other organisms including *Drosophila*, *C. elegans*, *Trypanosoma brucei* and *S. mansoni* [164, 165, 166, 167, 168]. It can also not be ruled out that since many enzymes and inhibitors other than just cysteine proteases and cystatin are involved in the moulting process [169, 170], they probably compensate for the loss of cystatin. In the recovered adult *A. viteae* worms which were treated with AvCys dsRNA no differences were observed in the transcript amounts or activity of cystatin as compared to the control worms.

As compared to *A. viteae*, the genome of the free-living nematode contains two cystatins, *Ce-cpi-2a* (R01B10.1) and *Ce-cpi-1* (K08B4.6). Though there are no reported RNAi phenotypes associated with any of these genes in the recently published genome-wide screens (www.wormbase.org), the cystatin, CPI-2a, was functionally characterised in the deletion mutant *cpi-2a*(ok1256) [127]. The authors [127] showed that CPI-2a has an essential regulatory role during the oogenesis and fertilization. They suggest that CPI-2a regulate the proteolytic processing of molecules within the gonadal sheath cells, directly or indirectly which are essential for proper cell-cell signalling activities of the gonadal sheath cells surrounding during normal maturation and ovulation processes. To determine if knockdown of cystatin in the adult stages would have any effect on ovulation or development of microfilariae in *A. viteae,* adult female worms were treated with AvCys dsRNA by soaking. However, unlike in *C. elegans,* no detrimental effect was observed on the ovulation or development of eggs and microfilariae suggesting that AvCys does not play an essential role at this stage. Therefore, it can be concluded that cystatin of the free living *C. elegans* and that of the parasitic *A. viteae* play different roles in their developmental life cycles.

4.2.2 Essential role of chitinase in moulting of L3 and hatching of microfilariae

Chitinases are attractive targets for intervention in filarial diseases since they are associated with the degradation of chitin, a polysaccharide which is present in nematodes but not found in vertebrates. Although chitinases of several filarial nematodes have been described at the molecular level [51, 52, 49, 50], relatively little is known about the function of these proteins during the life cycle of the parasites. An earlier study (Tachu B, PhD thesis, 2006) has shown that though *A. viteae* genome has three chitinase genes, only one is expressed.

The function of chitinase gene I of *A. viteae* was analyzed by targeting it using RNAi. In *A. viteae* L3 larvae treated with chitinase full length dsRNA (AvChifl dsRNA) or the serine threonine rich 3' region of chitinase (AvChi3' dsRNA) moulting was inhibited as compared to the control Mal dsRNA treated larvae. Moulting to the L4 stage was inhibited in 90% of the L3 larvae. However, chitin has so far not been described as a component of the cuticle of nematodes, and even in the well studied nematode like *C. elegans* it has only been described as a component of the pharynx [171, 172]. As it is unlikely that remodelling of the pharynx alone requires large amounts of chitinase, it is probable that the nematode cuticle contains chitin-like polysaccharides or glycosylated proteins that are target of chitinase. Studies on the substrate specificity of the enzyme would answer this question. Ultrastructural studies showed that the chitinase of *A. viteae* L3 is stored within cells of their oesophageal glands [49]. These glands open into the oesophagus, but their contents also reach the surface of the worms via a system of lacunae [173]. This localization in L3 and the form of release are compatible with the role of chitinase in remodelling of the L4 cuticle and casting of the L3 cuticle, with a concomitant release into the host tissues. A substantial release of chitinase by *A. viteae* L3 has been observed [112] suggesting that chitinase might also act on host components, for instant by degrading extracellular matrix, thus facilitating the migration of the larvae.

These phenotypic differences were correlated with a decrease in chitinase transcript levels which was quantified by real-time PCR. Worms which were treated with AvChi3' dsRNA had 97% less transcripts than the control. Surprisingly in worms that were treated with AvChifl dsRNA, 43 times more transcript of chitinase was observed. The reason for this upregulation is unknown and may be a non-specific

effect. A similar response of upregulation was observed in *H. contortus* when the gene vacuolar ATPase was targeted by RNAi [134]. Recently, Long-Cheng Li et al., [174] have shown that some small dsRNA (21nt) molecules targeting the promoter sequence of the mammalian protein E-cadherin, induced high levels of gene expression. It is probable, that the dsRNA of full length of chitinase of *A. viteae* (AvChifl dsRNA) when cut with the DICER results in some small dsRNA molecules which have significant homology to the promoter region and thereby induces activation of expression or upregulation of transcription of chitinase at the L3 stage as observed in this study.

This and earlier studies have observed that chitinase is found in the developing microfilariae just before hatching. This suggests that chitinase plays a role in the hatching of microfilariae from their egg shells which contain chitin. To confirm this function, chitinase gene I in adult female worms was targeted by RNAi using the soaking method. Female and male worms in groups of three and five respectively were treated with 75pmol / 100 μl of AvChifl, AvChi3' or Mal dsRNAs. Female worms treated with AvChifl and AvChi3' dsRNA had a high mortality rate with 80 and 60 % of the worms dying after the incubation period and the control group worms were lethargic. The high mortality in female worms, which was in stark contrast to the vitality of male worms, suggests that disturbance of the chitin metabolism in the female worms has gender specific fatal consequences beyond the inhibition of egg shell casting. Therefore, it cannot be excluded that chitinase plays an unknown role in the metabolism of female worms. The lethargicity of the control worms is attributed in other studies [61] to the toxicity of high concentration of dsRNA and was confirmed by the recovery of the worms when transferred to fresh medium.

A. viteae are ovo-viviparous, meaning that the egg shells are shed shortly before birth and the microfilariae are released into the blood stream of the host unsheathed. In this study, adult worms which were treated with AvChifl or AvChi3' dsRNA released microfilariae, 50 to 75 % of which were unhatched or still in their egg shells. This confirms that chitinase is essential for hatching of microfilariae and also the persistence of RNAi in adults even in the absence of dsRNA for up to 5 days. The silencing of chitinase by RNAi led to a significant reduction in the number of larvae released into the medium. However, not all worms were affected to the same extent

as observed by the large range in decrease (50 to 75%) in the number of microfilariae released. In *L. sigmodontis* RNAi studies, the variation in the release of microfilariae among adult worms was ascribed to the differences in the thickness of the cuticle between individual worms [175]. This in turn could contribute to an inefficient uptake of dsRNA from the surrounding medium, especially if uptake is strictly passive. These results prove that chitinase is relevant for shedding the egg shell within the uterus. This process seems to be very sensitive, as a clear morphological effect was seen in spite of a reduction of chitinase mRNA by only 19%. Nonetheless, this small decrease in transcript levels induced a clear phenotype of inhibition of hatching of microfilariae.

This study, for the first time, shows that *A. viteae* is amenable to RNAi and the existence of RNAi pathways suggesting that RNAi can be used to further identify potential targets for intervention strategies. Moreover, the suggested functions of cystatin and chitinase in moulting and hatching of microfilariae were confirmed.

4.3 Temporal expression of cystatin and chitinase

In this study, the regulation of cystatin and chitinase during the life cycle of *A. viteae* was analyzed. *A. viteae* cystatin is an excretory-secretory protein which is suggested to be secreted during the microfilarial, infective L3 and adult stages of the life cycle. Analysis was done by real-time PCR to determine level of expression of cystatin during all the stages (uterine microfilaria, blood microfilaria, L2, infective L3, young L4, late L4, young adults and mature adults) of the life cycle.

Cystatin transcripts were found in all stages of the *A. viteae* life cycle however, the level of expression was regulated. The maximum amount of cystatin transcripts was observed in the blood microfilarial stage and the minimum in the adult male stage. The transcript levels at the L3 stage, where cystatin was determined to play a role in moulting, was less than that in the blood microfilarial stage but higher than that of in adult stages. In the free living nematode *C. elegans*, where cystatin (CPI-2a) was determined to play an essential role in embryogenesis [127], expression of CPI-2a transcript was different from that in *A. viteae*. In *C. elegans* expression of CPI-2a transcript is elevated 2h prior to every moulting stage coinciding with the expression of cathepsins [127].

The difference in the developmental expression of cystatin in the free living *C. elegans* and in the parasitic *A. viteae* is probably due to the different roles cystatin play in these nematodes. While *C .elegans* cystatins are protease inhibitors involved in embryogenesis, moulting or feeding, *A. viteae* cystatin has evolved as an immunomodulatory protein interfering with the proliferation of host T cells in addition to its protease inhibitor functions during moulting. The microfilariae of *A. viteae*, which are in a state of arrested development, circulate in the peripheral blood of the host and do not moult until it is taken by the arthropod host. The microfilariae therefore are exposed to a constant array of host immune responses in the blood. Therefore, the upregulation and secretion of an immunomodulatory protein like cystatin ensures the survival of the parasite in the host. This suggestion is further complemented by observations that *A. viteae* cystatin induces a Th2 kind of response and that microfilaremic state in individuals with filariasis is associated with a Th2 response [176]. Moreover, the appearance of microfilariae during natural infection is most closely associated with the suppression of the host immune responses [177]. In *B. malayi*, TGF-2 homologue suggested to have immunomodulatory properties is also maximally expressed in the blood microfilarial stage [117].

It was observed that the amount of cystatin transcripts dropped when the blood microfilariae are taken up by the arthropod host and develops to the L2 and L3 stages. Cystatin could also play a role in the moulting of the L2 to the L3 stage in the arthropod since the transcript levels of cystatin at both these stages were the same. After the mammalian host, *Meriones*, is infected with the L3 larvae, the amount of cystatin transcripts decreased as the larvae developed to the L4 stage. As the L4 larvae sexually differentiated into adults, the amount of cystatin transcripts varies with an increase in females and a decrease in males. The higher amounts of cystatin in gravid adult female worms could be due to the uterine microfilariae or because it is involved in embryogenesis as in the free living *C. elegans* [127].

Chitinase gene I transcript was observed during all the stages of the life cycle of *A. viteae* though, at different levels. Expression of chitinase was observed in the intrauterine microfilariae after which it decreased as the microfilariae were released into the blood stream. *A. viteae* microfilariae are born ovo-viviparously, shedding the

egg shell shortly before birth. Chitinase of *A. viteae* is involved in the hatching of microfilariae as determined by RNAi experiments. Chitinase degrades the chitinous oolemma surrounding the developing eggs in utero as observed earlier in *O. gibsoni* [178].

Chitinases have been also been suggested to play a role in parasite transmission in *B. malayi*, whose microfilariae are enclosed in chitin sheaths. *B. malayi* microfilariae not expressing chitinase could not penetrate the arthropod vector [179]. It is hypothesised that lectins or agglutinins in the arthropod midgut binds to the microfilariae and reduces their infectivity. Chitinase promotes infectivity by digesting its substrate and releasing N-acetylglucosoamine (GlcNAc) that saturates the lectins and reduces their interaction with microfilariae. However, *A. viteae* microfilariae are not sheathed unlike the *B. malayi* microfilariae. Therefore, the role of chitinase in the parasite transmission in *A. viteae* cannot be assumed.

After the blood microfilariae are taken up by the arthropod they develop to the L2 and L3 stages. As the microfilariae developed to the L2 and L3 stages expression of chitinase increased, with the maximum in the L3 stage. These results were corroborated by Western blots (Tachu et al., 2006 submitted). This raises the question of the role of chitinase in the L2 and L3 stages in the arthropod host. In *O. volvulus*, lysis of the peritrophic membranes, a chitinous structure in the midgut, in *Simulium* vectors [180, 181, 182] by chitinase is essential for migration of the larvae from the midgut to the haemocoel. However, *A. viteae* tick vectors do have such peritrophic membranes [183] and it is therefore not known whether *A. viteae* larval migration in the tick vector requires chitinases.

As the L3 larvae developed to the L4 in the mammalian host, *Meriones*, the amount of chitinase transcripts was observed to decrease. Our RNAi studies showed that chitinase is essential in the process of moulting as inhibition prevents 90% of L3 to moult. Our studies can, however, not answer the question why in late L4 stages, that have to moult into adult worms within a few days, the amount of chitinase transcripts decrease, given that the process of moulting should not be principally different from the moulting of the L3 to L4. At the L4 stage, the pharynx becomes functional and the pharyngeal cuticle contains chitin. Morever, *B. malayi* was

demonstrated to have chitin synthase in L4 stages [184]. This would be compatible with the fact that *A. viteae* L4 require chitinase to regulate the synthesis of chitin in the pharyngeal cuticle. However, in filariae, the pharynx and intestinal tract do not appear to play a role in nutrient uptake [185, 186]. Further knockdown studies at the L4 stage would determine the role of chitinase at this stage. As the L4 developed to adult stages, the amount of chitinase transcripts increased with female worms having more transcripts than males. As determined by RNAi, in females, chitinase was observed to be involved in the hatching of microfilariae. Earlier ultrastructural studies have revealed that immature uterine microfilariae bear chitinase within their cuticle, while the protein appears on the surface of the cuticle when maturation is completed [164]. Our immunofluorescence data are in line with this observation showing that the protein can be detected by antibody labelling on the surface of mature, but unhatched microfilariae, while it is not detected in earlier (embryos) or later (released microfilariae) stages.

Chitinase transcripts were also observed in *A. viteae* adult male worms though the quantity was not as much as in female worms. This raises the question as to what the function of chitinase could be in male worms, which is obviously not for hatching of microfilariae. The plant parasitic nematode, *Heterodera glycines*, secretes chitinase (Hg-CHI-1) into the plant tissue suggesting that the natural substrate must not be chitin, since this polymer does not occur in plants [187]. In addition, the expression of Hg-CHI-1 was not observed in the eggs indicating that this chitinase does not play role in egg hatching. Moreover, the expression of an 18 glycosyl hydrolase gene (chitinase) in *C. elegans* was detected in the life stages not containing chitin, including high expression in dauer larvae [58]. Therefore, the presence of chitinase transcripts in the adult male stage of *A. viteae* supports the notion that chitinases cleave substrates other than chitin.

4.4 *A. viteae* cystatin as a vaccine candidate

Several studies offer credence to the view that development of protective immunity is a possibility in filariasis. This stems from the observation that only a certain percentage of people living in endemic areas acquire filarial infection / disease [188, 189, 190]. An understanding of the protective mechanisms in putatively immune individuals and in animal models will help in the targeted development of

vaccines. Some studies have suggested that protective immunity in putatively immune individuals is due to diminished specific immunoglobulin (Ig), IgG subclass responses and an enhanced production of IL-2 and gamma interferon in response to adult worm antigens. This suggests that protective immunity is dependent on Th1 responses [188, 191, 192, 193]. In other studies, protective immunity was suggested to be due to mixed Th1 and Th2 responses [194]. In animal models of filariasis, it has been observed that antibodies directed towards the surface of L3, the larval moulting [195, 196, 197] and the surface of microfilarial sheath [198] play a role in limiting larval development. Effector cells such as macrophages and eosinophils [198, 199, 200, 201, 202], cytokines such as IL-4 and IL-5 [203] and cytotoxic molecules such as nitric oxide are also involved in enhancing microfilarial clearance. Moreover, immunisation with irradiated L3 induced protective immunity by killing challenge larvae [204, 205] and was dependent on IL-4 and IL-5 demonstrating Th2 immunity.

Filarial cystatin was considered as an attractive vaccine based on the following observations: (i) Cystatin is expressed in the infective L3 stage, (ii) It is an immunomodulatory protein and is, therefore, probably required for the infectivity and (iii) *O. volvulus* cystatin was identified by antibodies found in PI individuals or immunised animals [102].

In this study, recombinant *A. viteae* cystatin expressed in *E. coli* was used for immunisation studies with STP or alum as adjuvants. No significant reduction in worm burdens was observed in either group. This was different from the immunisation studies with *O. volvulus* cystatin with alum, which induced a 34 % reduction in worm burdens [102]. However, in the case of *O. volvulus* cystatin the recombinant protein was expressed in yeast. This could probably explain the differences in protection rates observed. A similar discrepancy was also observed in the case of immunisation studies of *B. malayi* with the basement membrane collagen epitope, AP2. While native AP2 induced significant reduction in adult worm burden, no protection was observed with *E. coli* expressed protein [206]. *E. coli* derived proteins might be unsuitable since they lack critical epitopes owing to improper folding of proteins or absence of post-translational modifications.

To enhance the protective efficacy, cystatin was also used as a DNA vaccine in this study. DNA immunisation is efficient against a diverse range of pathogens

[207]. Moreover, while protein immunisation is often restricted to major histocompatibility complex class 2-directed responses, MHC class I and II presentation of antigen expressed by DNA transfected cells can elicit both cytolytic T cell and antibody responses. DNA vaccines have been shown to induce protective immunity against the intracellular protozoan parasites, *Plasmodium falciparum* [208, 209] and *Leishmania major* [210], where protection is mediated by Th1-like responses associated with interferon-γ stimulation. In *A. viteae*, immunisation with a DNA vaccine encoding tropomyosin induced a partial protection of 50 % [211]. The authors observed this effect only when a Th1 polarized immune response was elicited, augmented by aluminium phosphate adjuvant, against challenge infection. Similarly, a partial protection against *O. volvulus* infection in mice was obtained when immunised with DNA coding for chitinase [212]. In this study, DNA vaccine encoding cystatin of *A. viteae* was used for immunisation experiments. Though, the first experiment showed about a 50% reduction in worm burdens as compared to the control groups, the results were not reproducible. In the second experiment only 23% reduction in worm burden was observed. Such inconsistencies in the induction of protection might be due to various factors one of which is differences in the transfection rates of myocytes after intramuscular injections. Immunological studies have demonstrated that gene transfection and subsequent activation of dendritic cells are key events in the development of immunity following DNA vaccination [213]. These antigen-presenting cells (APCs) process peptide epitopes at the expressed antigen in context of both class I and class II MHC molecules resulting in the induction of cytotoxic T lymphocytes (CTL) with the help of CD4+ T cells [214, 215]. Therefore, high transfection of APCs is essential for an efficient DNA vaccination.

Plasmid DNA encoding *A. viteae* cystatin was encapsulated in cationic liposomes to enhance the transfection of APCs and thereby enhance expression of the antigen. This would lead to a Th1 kind of response and a better protection rate than using naked plasmid as a vaccine. However, it led to only 29% reduction in worm burdens as compared to the controls. Moreover, the results were not statistically significant. Differences were observed neither in the microfilaria burden nor in the sizes of adult worms in animals immunised with liposome encapsulated DNA as compared to the control groups. It can however not be definitely concluded that vaccination with either naked plasmid DNA or liposome encapsulated DNA

confers no protection to challenge. The experiments need to be repeated with probably a different approach to increase T-cell responses to vaccination by heterologous prime-boost immunisation strategy.

4.5 OUTLOOK

Investigations on the molecular mechanisms of genes required for parasitism in filarial nematodes is essential to develop novel therapeutic and preventive strategies against filariasis. In this study the proteins, cystatin and chitinase of the rodent filarial nematode *Acanthocheilonema viteae* were characterised.

Using *Caenorhabditis elegans* as a heterologous system it was determined that *A. viteae* cystatin is mostly expressed and/or stored in the pharyngeal and rectal gland cells suggesting that it plays a role in moulting. Knockdown studies of cystatin by RNAi further confirmed that moulting of L3 to L4 was delayed significantly, although the effect was transient. Quantification of cystatin through the whole life cycle of *A. viteae* determined that it maximally expressed in the microfilarial stage which is fully exposed to the host immune responses. It will be of interest to analyse the immune response of the host infected with *A. viteae* larvae in which cystatin is knocked down. This would determine the importance of the host immunomodulatory effects of *A. viteae* cystatin for infection.

Furthermore, this study determined the essential roles of chitinase in the developmental cycle of *A. viteae*. Chitinase plays an essential role in moulting since knockdown of chitinase in the L3 larvae inhibited moulting thereby killing them. However, L3 larvae do not contain chitin and further studies would have to determine the molecular mechanism and substrate of chitinase during the moulting process. Knockdown of chitinase in adult female worms also led to the release of unhatched microfilariae determining its essential role in the degradation of the chitinous egg shells. Future studies to determine the other substrates of chitinase will further the understanding of its molecular mechanisms.

Taken together this study demonstrates that cystatin and chitinase play important essential roles in the development of *A. viteae* and therefore are attractive intervention targets.

5 METHODS

5.1 Parasitological methods

5.1.1 Maintenance of the life cycle of *Acanthocheilonema viteae*

The life cycle of *A. viteae* was maintained essentially as described by Lucius and Textor [216]. Briefly, the infective L3 stages of *A. viteae* were obtained from infected *Ornithodoros moubata* ticks and used to infect *Meriones unguiculatus* subcutaneously. The L3s develop to the L4 and subsequently to the adult male and female stages. The female worms release microfilariae into the peripheral blood. The peripheral blood of infected *Meriones* was used to infect ticks where the microfilariae develop to the L2 and subsequently to the infective L3 stages.

5.1.2 Quantification of microfilarial load in blood of jirds

Infected *Meriones* were anaesthesized with ketamin:xylazin:normal saline (1:1:8) and bled from the retro-orbital sinus using a heparinised glass capillary tube. The blood (44.7 µl) was mixed with 100 µl Teepol (10% in H_2O) and the microfilarial load was estimated using a Fuchs-Rosenthal counting chamber.

5.1.3 Isolation of filariae

5.1.3.1 Isolation of adult *A. viteae* from *Meriones unguiculatus*

Meriones were anaesthetized and fully bled from the retro-orbital sinus. Following dissection of the jirds, adult *A. viteae* were isolated from the subcutaneous and intramuscular tissues, the inguinal and subscapular regions and some times in the thoracic chamber. Animal carcasses were then incubated in normal saline (0.9% NaCl) overnight to allow the rest of the worms to wander out. The female and male worms were collected separately in complete RPMI [RPMI 1640 medium supplemented with pencillin and streptomycin (final concentration of 1unit/ml), L-glutamate (final concentration of 2mM) and fetal calf serum (final conc. of 10%)].

5.1.3.2 Isolation of L3 stages from the vector Ornithodoros moubata

L3s of *A. viteae* were isolated from infected *O. moubata*. The ticks were cut medially and briefly rinsed in a petri dish with incomplete RPMI (iRPMI) to remove rests of blood meal and loose tissue. The ticks were incubated in warm iRPMI solution for 1 h for the L3s to migrate. The L3s were washed in iRPMI thoroughly for use in further experiments.

5.1.3.3 Isolation of late L3s from Meriones unguiculatus

Jirds were infected subcutaneously with L3s which were isolated from *O. moubata*. After 5 days, the *Meriones* were sectioned into pieces of 1 sq cm and immersed into warm cRPMI to let the late L3s migrate out from the muscles. The late L3s were washed in cRPMI and used either for isolation of RNA or for observing the moulting to L4.

5.2 *Caenorhabditis elegans* methods

5.2.1 Maintenance of *Caenorhabditis elegans*

The life cycle of *C. elegans* was maintained as described earlier in *C.elegans* - A practical approach [217]. Briefly, *C. elegans* wild type N2 strain was maintained monoxenically on nematode growth medium (NGM) agar plates with *Escherichia coli* OP50 strain as food source at 25°C. *E. coli* OP50 strain was used since it is a uracil auxotroph whose growth is limited on NGM plates and therefore allows for easier observation of the worms. For promoter and expression studies, GE24 strain of *C. elegans* was used. They are mutant for the *pha-1* gene which is a transcription factor required for the development of the pharynx. At 25°C they are zygotic embryonic lethal and the pharynx fails to undergo late differentiation and morphogenesis. However, the worms develop normally at a lower temperature of 15°C.

5.2.1.1 Decontamination and synchronisation of *C. elegans*

The *C. elegans* worms were cleaned of OP50 or contaminating bacteria by treating with bleach. Agar plates that have 100,000 gravid adult hermaphrodites were washed off the plate using M9 medium to a total volume of 3.5 ml. To this 0.5 ml of 5 M NaOH and 1 ml of 5% solution of sodium hypochlorite were added. The bleach solution containing the worms was vortexed vigorously every 2 min for a total of 10 min. It was then centrifuged at 2000 rpm for 30 sec to obtain a pellet of the intact

eggs while all the other stages and bacteria are digested. The eggs were washed extensively in M9 medium and seeded onto NGM plates. The same method was used to obtain a synchronised culture of *C. elegans*.

5.2.1.2 Removal of bacteria through sucrose gradient

To obtain *C. elegans* worms without the contaminating bacteria, the worms were purified through a sucrose gradient. NGM plates containing a mixed culture of *C. elegans* were washed off with M9 medium and spun down at 1200 rpm to pellet down the worms. The worms were resuspended in cold M9 medium. To this an equal volume of ice cold 60% sucrose was added, mixed thoroughly and centrifuged at 2500 rpm at 4°C for 20 min. The debris and bacteria formed a tight pellet while the healthy *C. elegans* worms formed a ring at the cap of the tube. The ring of worms was transferred to a fresh tube and washed twice with M9 medium to obtain a pellet of a mixed population of worms.

5.2.1.3 Preparation of stocks

For preparing stocks of *C. elegans,* freshly starved L1 - L2 worms were washed off plates with S-Buffer. To this, an equal volume of freezing solution was added and was aliquoted into cryovials. They were frozen slowly in –80°C. The stocks were thawed by inverting a cryotube onto a NGM agar plate and let to recover at 25°C in case of wild type worms or at 15°C in the case of *pha-1* mutants.

5.2.2 Transformation

C. elegans worms were transformed to express *A. viteae* genes and to analyse the expression patterns of genes using reporter constructs. Stable transformation was achieved by microinjection and worms were transiently transfected by the particle bombardment method.

5.2.2.1 Microinjection

The *C. elegans* worms were transformed by microinjection of plasmid DNA into the syncytial gonad of the worms. The injection mixture of 20 μl consisted of the plasmid constructs and co-injection marker at a final concentration of 100 μg/ml. The injection mixture was centrifuged at 14,000 rpm for 10 min and the topmost 3 μl was transferred into a new tube for use in injection. This was loaded into microinjection needles (Femtotips, Eppendorf) such that no air bubbles were introduced. Young

adult hermaphrodite *C. elegans* worms were pre-selected onto cold NGM agar plates with no bacteria. They were immobilized on dried agarose pads with the dorsoventral axis of the worm parallel to the surface of the coverslip and overlayed with a drop of light mineral oil. The microinjector was mounted on a micromanipulator and the worms on slides were placed on an inverted microscope to aid the microinjection. The loaded femtotips were penetrated into the gonad of the worms and the injection was done at a pressure of 460 psi until the gonad was completely filled (Fig. 5.1) with the injection buffer containing the construct and the marker plasmid. Worms were recovered with M9 medium and transferred onto NGM plates seeded with OP50 bacterial lawn. Transgenic worms were selected based on the phenotype ascribed to them by the marker plasmid. In the case of promoter studies, the marker plasmid, pRF4, rendered the wild type worms a roller phenotype. For expression studies, the marker plasmid, pBX, rendered the *pha-1* mutant worms the ability to survive at 25°C.

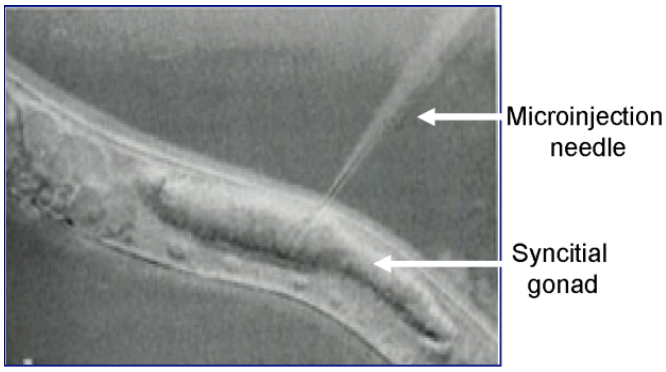


Figure 5.1 Microinjection of *Caenorhabditis elegans*

Young hermaphrodite *C. elegans* worms were transformed by microinjecting plasmid DNA into the syncitial gonad.

5.2.2.2 Particle bombardment

The particle bombardment techinique, developed by Sanford et al., involves accelerating DNA-coated particles (microprojectiles) directly into intact tissues or cells. A burst of helium gas accelerates a macrocarrier, upon which DNA-coated microcarriers have been dried.

C. elegans worms were washed off NGM plates and cleaned through a sucrose gradient as mentioned earlier. About 10,000 worms were placed in the

centre of an NGM plate placed on ice so that worms do not move. The microcarriers were prepared as described below. Gold powder (30 mg) was suspended in 1 ml of 100% ethanol in an eppendorf tube and vortexed for 15 min. After a short centrifugation of 5s, the gold pellet was washed with 1ml of 70% ethanol and centrifuged. The pellet was suspended in 500 μl of glycerol to a final concentration of 60 mg/ml. To coat the gold particles with plasmid DNA, 5 μg of plasmid was added to 17 μl of gold particles along with 17 μl of 0.1 M $CaCl_2$ and 7μl of spermidine and vortexed vigorously for 5 min. The reaction mix was centrifuged for 2 s and the pellet was washed with 200 μl of 70% ethanol followed by wash with 200 μl of 100% ethanol. The coated gold particles were resuspended in 20 μl of 100% ethanol and coated onto the centre of macrocarriers. Rupture discs of 1550 psi were used to bombard the *C. elegans* worms (*pha-1* or WT) with the coated microcarriers under pressure. The bombarded worms were allowed to recover at 15°C or 25°C for 24 h and the healthy worms were transferred to fresh NGM plates. The fluorescence in transfected worms was observed with a confocal microscope.

5.2.3 Integration of extrachromosomal arrays

The injected DNA in transgenic *C. elegans* worms form large extra-chromosomal arrays which may induce silencing of the genes and mosaic expression. Therefore, the extra-chromosomal arrays were integrated into the genome by mutagenesis. Transgenic L4 worms containing the arrays were placed on to a fresh NGM plate and irradiated with 3000 rads of gamma rays. The irradiation resulted in about 30% lethality. Three irradiated worms (P0) were transferred on to individual plates. From these plates, some of the F1 progeny was transferred to fresh individual plates. After 6 days, the F3 progeny which showed 100% transmittance of the transformation marker, survival at 25°C, were picked to establish transgenic lines.

5.3 Cell culture methods

5.3.1 Maintenance of mammalian cells

COS7 (African green monkey kidney cell type) and HeLa cells (human epithelial cells from a fatal cervical carcinoma) were maintained in complete RPMI 1640 under standard tissue culture conditions at 37°C and with 5% CO_2. When the culture flasks were confluent, the cells were washed with 1X PBS and treated with Trypsin/EDTA solution at 37°C for 2 min so that they are not adhered to the flask

bottoms. The cells were centrifuged and washed with complete RPMI to remove trypsin and a portion of this was resuspended in complete RPMI to be passaged.

5.3.2 Preparation of stocks

The mammalian cells were suspended in complete RPMI to obtain a concentration of 10^7cells/ml. To this, sterile dimethyl sulfoxide (DMSO) was added to a final concentration of 10% and frozen slowly in a styrofoam box at -80°C and later transferred to liquid N_2 for long term storage.

5.3.3 Transfection of COS7 and HeLa cells

Mammalian cells were transfected using either lipofectamin reagent (Invitrogen) or Fugene reagent (Roche). Cells were plated out (10^5 per well) in 12-well plates 24 h prior to transfection. For transfection with the lipofectamin, 2 µg of the desired plasmid and 8 µl lipofectamin were resuspended in 100 µl serum-free medium separately. After 30 min incubation at room temperature (RT) the solutions were mixed and incubated for another 30 min at RT. To this, 800 µl serum-free medium was added and applied to the cells. After 6 h of incubation under standard tissue culture conditions the transfection reagent was removed and the cells were maintained in complete RPMI 1640 before microscopic examination after 48 h.

For transfection with Fugene, 1 µg of the desired plasmid and 3 µl of the reagent was added to 100 µl of serum free RPMI. This was allowed to stand at room temperature for 15 min such that the plasmid DNA is complexed with the liposomal reagent and was added to the plated cells. The expression was observed after 24 - 48 h by microscopy or RT-PCR or western blot.

5.4 Protein analytical methods

5.4.1 Determination of protein concentration

Protein concentration was determined using the BCA kit (Pierce, Rockford, USA). The basis of this reaction is the biuret reaction: reduction of Cu^{2+} to Cu^{1+} by a peptide bond under alkaline conditions. Chelation of two molecules of bicinchoninic acid (BCA) with the cuprous ion (Cu^{1+}) produces a water soluble complex, whose solution has a deep purple colour and an absorbance at 562 nm. A linear standard

curve was made with 0.05 to 2 mg BSA and concentrations of the unknown protein samples were determined using the standard curve.

5.4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Analytical polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as described by Laemmli (1970). Samples were heated for 5 min at 100°C in sample buffer. Samples were applied at volumes of 10-30 µl to a 12.5% slab gel cast in a mini-gel apparatus. The electrophoresis was performed at 20mA with electrode buffer. The run was stopped when the bromophenol blue dye front reached the end of the gel. Protein bands were observed by staining the gel with Coomassie Brilliant Blue R250 and the gels were dried for a permanent record.

5.4.3 Inhibition assay of cystatin

To assess the inhibitory activity of cystatin against papain, a spectroflourometric assay was performed. The colorimetric substrate benzoyl-DL-Arg-p-nitroamilide (BAPNA) was used to assay papain in the enzymatic assay buffer. The reactions were performed at room temperature for 30 min and stopped by the addition of 200 µl of 10 M acetic acid. Released *p*-nitroanilide was measured by a spectrophotometer at 405 nm. The assay was standardized for total antigen of *A. viteae* worms by varying the concentration of papain from 0.6 µM to 6.6 µM and the concentration of total antigen ranging from 10 µg/ml to 100 µg/ml. The best inhibition of papain was obtained at a papain concentration of 0.6 µM and an antigen concentration of 10µg/ml. Therefore, these concentrations were used for further inhibition assays of cystatin.

5.5 Immunochemical and immunological methods

5.5.1 Western blot

Proteins separated by SDS-PAGE were immobilised onto nitrocellulose membranes by Semi-Dry electrophoretic transfer. A transfer cassette was assembled using Whatmann paper cut to the size of the gel soaked in Semi-Dry transfer buffer followed by nitrocellulose membrane (pore size 0.2 µM), SDS-PAGE gel and finally Whatmann paper pieces. The cassette was introduced between the electrodes so that the gel with the protein was towards the negatively charged cathode and the

nitrocellulose membrane towards the positively charged anode. A constant current of 80 mA was then applied across the cassette at room temperature for 45 min. The membrane was blocked by incubation for 45 min in 5% skimmed milk powder in PBS or 2% BSA. Nitrocellulose membranes with immobilised proteins were incubated with a primary protein specific antibody for 1 h at room temperature followed by three 5 min washes in wash buffer (PBS, 0.02% Tween 20). The membrane was then incubated in a secondary conjugate antibody for 1 h followed by washes as above. Detection was done by using a substrate for the alkaline phosphatase enzyme conjugate, 5-Brom-4-chlor-3-indolylphosphate (BCIP) and tetrazolium chloride (NBT) in alkaline phosphatase buffer. All antibodies were diluted in the blocking solution.

5.5.2 Bleeding of animals for production of sera

Mice and jirds were bled from the retro-orbital sinus using a microhaematocrit capillary, and the blood was stored at 4°C overnight. Blood samples were centrifuged at 10,000 rpm at 25°C for 20 min to separate the sera from blood clots. Sera were stored in 100 μl aliquots at -20°C.

5.5.3 Enzyme linked immunosorbant assay (ELISA)

Antibody responses to *A. viteae* cystatin in the sera of immunized *Meriones* were determined by ELISA. Round bottom 96-well plates were coated with 100 ng/well of recombinant cystatin in carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing three times with PBS containing 0.025% tween (PBST), the wells were blocked for 30 min with 100 μl 3% BSA/PBS at 37°C. After subsequent washing with PBST, sera were added in a dilution ratio of 1:80 in blocking solution and incubated for 2 h at 37°C. Subsequently, the plates were washed again with PBST and incubated with the secondary antibodies: goat anti-mouse IgG or IgM or rabbit anti-mouse IgG1, IgG2a, IgG2b and IgG3 conjugated to alkaline phosphatase in a dilution ratio of 1:3000 in 3% BSA/PBS. After 1 h incubation at 37°C the plates were washed again and the substrate (5 mg p-nitrophenyl phosphate disodium) dissolved in 10 ml carbonate buffer with 1 mM $MgCl_2$ was added. The reaction was stopped with 0.5 N NaOH and the absorbance at 405 nm with the additional reference filter at 630 nm was measured. All assays were performed in triplicates.

5.5.4 Immunostaining of *A. viteae* eggs

The intrauterine content of *A. viteae* adult female worms was stained to analyse the expression of chitinase. *A. viteae* embryos were released from the adult female worms by cutting them into small pieces in RPMI and centrifuged to obtain a pellet. The embryos were fixed by treating with 200 µl of 4% formaldehyde for 5 min followed by 5 min in cold methanol. The embryos were centrifuged and washed thrice with 500 µl of PBS with 0.5% tween 20 (PBST). The blocking was done with 100 µl of 10% FCS for 30 min followed by incubation with 100 µl of 1:100 dilution of primary antibody (monoclonal anti-Chitinase antibody) in 10% FCS for 2 h. The embryos were washed with 500 µl of PBST twice and incubated with FITC-labelled anti-mouse secondary antibody diluted in a ratio of 1:500 in 10% FCS at 37°C in the dark. The embryos were washed thrice with PBST and suspended in 50 µl of PBS and mounted on to slides for microscopy.

5.6 Molecular biology methods

5.6.1 Isolation of high molecular weight genomic DNA from *A. viteae* and *C. elegans*

About 200 mg to 1 g of adult *A. viteae* or *C. elegans* worms were snap-frozen in liquid nitrogen and ground to powder using a mortar and a pestle. Up to 100 mg of powdered worm material was suspended in 1.2 ml of digestion buffer containing Proteinase K (100 µg/ml), and incubated with shaking at 50°C for 12 to 18 h until the sample became viscous with a visible sludge. The samples were extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol. The aqueous phase was transferred each time with a wide-bore pipette (0.3-cm-dimeter orifice) into a new tube. The RNA was digested by the addition of 20 µg/ml RNase A and incubated for 1 h at 37° C. Genomic DNA was then precipitated by adding half volume of 7.5 M ammonium acetate and 2 (original) volumes of 100% ice-cold ethanol. Stringy precipitates of DNA were carefully transferred in to new tubes using a blunt spatula and washed twice with ample amounts of 70% ethanol (2 times the original volume). The DNA pellet was air dried and dissolved in Tris-EDTA buffer. The concentration of the genomic DNA was determined by spectrophotometry and the DNA was analysed on a gel.

5.6.2 Electrophoresis and detection of DNA on agarose gels

Agarose gel electrophoresis was used for the routine analysis of DNA. Agarose gels were cast in 1X Tris Acetate EDTA buffer containing 0.5 µg/ml ethidium bromide. The DNA samples (0.1 to 5 µg) were dissolved in DNA loading buffer and separated in agarose gels at 10 volts per cm. The concentration of the agarose gel was relative to the size of DNA fragments to be separated and was typically between 0.7 and 1.2%.

5.6.3 Isolation of DNA from agarose gels

Following electrophoresis, a DNA fragment was excised from agarose gels under UV transilluminator using a sterile scalpel. The DNA was purified using the NucleoSpin® Extraction Kit (Clontech) according to the manufacturer's instructions.

5.6.4 Isolation and concentration of DNA from aqueous solutions

5.6.4.1 Extraction with Nucleospin kit

The NucleoSpin DNA Extraction Kit (Clontech) was used according to the manufacturer’s instruction for the isolation of DNA from PCR reactions and other aqueous DNA solutions

5.6.4.2 Phenol chloroform extraction

To remove protein contaminants from DNA, the volume was adjusted to at least 300 µl with Tris EDTA (TE) buffer and extracted with an equal volume of Tris-equilibrated phenol:chloroform:isoamyl alcohol. The aqueous phase was extracted twice with chloroform to remove traces of phenol and the DNA was concentrated by ethanol precipitation.

5.6.4.3 Precipitation of DNA

Ice cold sodium acetate (pH 4.5) was added to a DNA sample to a final concentration of 0.3 M. To this sample 2.5 volumes of ice cold absolute ethanol was added. The sample was incubated at -70°C for 15 min and centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was washed in 70% ethanol, air-dried and re-suspended in an appropriate buffer.

5.6.5 Isolation of plasmid DNA

A single colony of bacteria was used to inoculate 3 ml of Luria Bertani (LB) medium with an appropriate antibiotic and was incubated at 37°C overnight. The overnight culture was centrifuged and plasmid DNA was isolated using the Nucleobond Plasmid isolation Mini kit (Clontech) according to the manufacturer's instructions. Large scale isolation of plasmid DNA was done from 200 ml of overnight bacterial culture using Nucleobond Plasmid Maxi kit (Clontech) according to the manufacturer's instructions.

5.6.6 Isolation of total RNA

Before isolation of RNA, all pipettes and working bench were made RNase free using 0.1M NaOH, 1mM ethylenediamine tetracetate (EDTA) and diethyl pyrocarbonate (DEPC) treated H_20. The electrophoresis tanks were washed with 0.5% SDS, DEPC treated H_20 and then with 70% ethanol. Snap frozen material was used for the isolation of RNA. *C. elegans* and *A. viteae* adult worms (1 to 20 mg) were ground in liquid nitrogen before being homogenised with the QIAshredder coloumns (QIAGEN). *A. viteae* L3 worms (1 mg) were lysed in the lysis buffer with a microcentrifuge tube pistil before being passed through QIAshredder. Mammalian cells were directly homogenised through QIAshredder. RNA was isolated from the QIAshredder lysates using RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. The integrity of the RNA was proofed by gel electrophoresis of the total RNA and the concentration was measured using the spectrophotometer.

5.6.7 Electrophoresis of total RNA

A 1% agarose gel was prepared in 1X formaldehyde agarose (FA) buffer and allowed to equilibrate in the running buffer for 30 min. Total RNA (4 μl) was mixed with 1 μl of 5X RNA loading buffer, heated at 65°C for 3 min, cooled on ice and loaded on to the gel. The gel was run at 80V to resolve the RNA. Two bands corresponding to the 28 S and 18 S ribosomal RNA, with no smear, was observed confirming the integrity of the total RNA.

5.6.8 Determination of the concentration of nucleic acids

The concentrations of DNA and RNA were measured using the Nanodrop Spectrophotometer (PeqLab) according to the manufacturer's instructions. The calculations were done by the instrument using the following formula.

1 unit of absorbance of dsDNA at OD_{260} = 50 µg/ml dsDNA
1 unit of absorbance of RNA at OD_{260} = 40 µg/ml RNA

The OD_{280} was also measured and the ratio between the two ODs indicated the purity of the DNA solution. For pure DNA,
$1.8 \leq$ absorbance at OD_{260} / absorbance at $OD_{280} \leq 2.0$

A value less than 1.8 indicates contamination with proteins or with aromatic substances like phenol, while a value greater than 2.0 indicates possible contamination with RNA.

5.6.9 Restriction digestion of DNA

DNA was digested at the optimal temperature of restriction enzymes according to the pipetting scheme as described below.

Component	**Amount**
DNA	(0.1 to 5 µg)
10X restriction enzyme buffer	2 µl
10X Bovine Serum Albumin	2 µl
Restriction enzymes	3 to 20 units
HPLC water	up to 20µl

Following digestion, the mixture was heated at 70°C for 15 min to inactivate the enzyme and purified using the Nucleobond PCR purification kit (Clontech).

5.6.10 Polymerase Chain Reaction (PCR)

Thermostable DNA polymerases were used for the amplification of DNA as described (Saiki et al., 1988 and Bej et al., 1991). Two types of PCR amplifications were used in this study: amplification of fragments about 3 Kb using the standard Taq

polymerase and amplification of larger fragments using Phusion high fidelity polymerase. The PCRs were set up and performed according to the following thermal profile.

Table 5.1 Pipetting scheme for the PCRs

Reagent	Taq polymerase	Phusion polymerase
Forward primer (10 pmol/ μl)	1 μl	5 μl
Reverse primer (10 pmol/ μl)	1 μl	5 μl
dNTP mix (2mM)	2 μl	2 μl
10X Reaction buffer with $MgCl_2$	2 μl	2 μl
DNA template	10-100 ng	10-100 ng
HPLC water	Up to 50 μl	Up to 50 μl
DNA polymerase	2.5 units	2.5 units

Table 5.2 Thermal Profile for the PCRs

Phase	Taq polymerase	Phusion polymerase
Denaturation	94°C, 1 min	98°C, 1 min
Denaturation	94°C, 1 min	98°C, 30 s
Annealing 30 cycles	53 – 60°C, 1min	53 – 60°C, 30 s
Elongation	72°C, 1-3 min	72°C, 1-3 min
Extension	72°C, 10 min	72°C, 10 min

5.6.11 Single worm PCR

C. elegans worms were analysed whether they were transgenic by single worm PCR. Single transgenic worms were picked from plates and placed in 2.5 μl of lysis buffer which contains Proteinase K. The tubes were snap frozen in liquid nitrogen for 10 min and incubated at 65°C for 1 h. The Proteinase K was inactivated by heating at 95°C for 15 min. The lysate was used as template for standard PCR with specific primers to confirm the presence of *A. viteae* genes in *C. elegans*.

5.6.12 Reverse Transcription PCR (RT-PCR)

Messenger RNA (mRNA) was reverse transcribed to cDNA using SuperScript III fReverse Transcriptase (Invitrogen). About 0.5 to 5 μg of total RNA was incubated with 0.5 μg of Oligo dT primer and 1 μl of 10 mM dNTP to a final volume of 10 μl at 65°C for 5 min. The reaction is then placed on ice for 1 min. To this, 2 μl of 10X

reaction buffer, 2 μl of 0.1M DTT, 1 μl of RNase Out, 1 μl of Superscript III RT and water to a final volume of 20 μl were added. The reaction was incubated at 50°C for 50 min followed by incubation at 85°C for 10 min to inactivate the enzyme. The synthesised cDNA was used as template for PCR reactions as mentioned above (Section 5.6.10).

5.6.13 Ligation

DNA fragments were ligated into vectors using the T4 DNA ligase. DNA fragments, PCR products and vectors were restriction digested as mentioned in section 5.6.9. The purified products, insert and vector were used for ligation. The ligation reaction was set up as follows.

Component	Amount
Vector	1/3 molar ratio of insert
10X ligation buffer	2 μl
Insert DNA	3 molar excess of vector
T4 DNA ligase	400 weiss units
HPLC water	upto 20 μl

The ligation reaction was incubated at 16°C overnight and 2μl was used for transformation of competent *E. coli* cells. For ligation of PCR products into the pGEM-T (Promega) vector, the same protocol as mentioned above was used. Insert DNA was used in 3 to 5 molar excess of vector and after incubation at 16°C overnight it was used for transformation.

5.6.14 Construction of plasmids

5.6.14.1 Constructs used for promoter studies of *A. viteae* cystatin and chitinase

For transfection of COS7 cells with the cystatin promoter, the pGEM-T plasmid containing the isolated genomic clone was used as a template in a PCR reaction to amplify the upstream genomic region using the plasmid-specific SP6 and a *Kpn*I linked clone-specific reverse primer (prCysR). The product was digested with *Sac*I and *Kpn*I and ligated into pEGFP-N1 (Clontech). The resulting plasmid was digested with *Sac*I and *Afl*II. The 1710 bp fragment encoding the potential cystatin promoter, EGFP and the SV40-poly A was purified by agarose electrophoresis and ligated into pSL1180 (Pharmacia). To construct a promoter-less negative control plasmid,

pEGFP-N1 was digested with *Eco*RI and *Afl*II. The 1010 bp fragment comprising the reporter gene and the SV40-polyA was then ligated into pSL1180. As positive control in transfection studies pEGFP-N1 was used. For transfection of COS7 cells with the chitinase promoter, the pBlueScript plasmid containing the genomic clone of chitinase gene I was used as a template in a PCR reaction to amplify the upstream genomic region using the primers prChiGfpF / prChiGfpR. This was cloned between sites *Bgl*II and *Sac*I into the vector pSL1180 – EGFP and was used to transfect COS7 cells.

To construct a cystatin promoter plasmid for the transformation of C. elegans, the pGEM-T plasmid containing the isolated genomic clone was used as a template in a PCR reaction to amplify the putative promoter region together with the first exon of cystatin using the specific primers (prCys95F / prCys95R). The product was cloned into the vector pPD 95.77 (Fire lab kit, 1995) digested with HindIII and PstI, in-frame with the reporter gene GFP to obtain pPrAvGFP. To construct a chitinase promoter plasmid for the transformation of C. elegans, the promoter sequence amplified with prChiGfpF / prChiGfpR was cloned upstream to the GFP reporter in the plasmid pPD95.77 digested with BglII and SacI to obtain pPrChiGFP. As negative controls, the vector pPD95.77, which had no promoter, was used.

5.6.14.2 Constructs used for expression of *A. viteae* cystatin in *C.elegans* and COS7 cells

The cDNA and genomic sequences of cystatin were amplified from A. viteae RNA and genomic DNA. The cDNA sequence and genomic sequence was amplifed using the primers 49CysF / 49CysR incorporating the His tag at the 3’ end. The amplicons were cloned downstream to a heat shock promoter (*hsp-16/42*) in the vector pPD49.83 digested with *NheI* and *KpnI* to obtain p49Av17c and p49Av17g. A heat shock promoter was chosen since it induces a high level of expression in a variety of tissues such as the gut, muscle, nerves and pharynx but with no germ line expression. The cDNA sequence of cystatin along with its 3’UTR amplified using the primers103CysF / 103CysR which included a His tag at the N-terminus was also cloned downstream to a constitutive promoter (*let-858*) in the vector pPD103.05 digested with NheI and SmaI to obtain p103Av173'. The *let-858* promoter drives the constitutive expression of cystatin to all somatic tissues. These constructs were used for expression of *A. viteae* cystatin in *C. elegans.*

5.7 Microbiological methods

5.7.1 Preparation of competent *E. coli*

Competent *E. coli* cells were prepared essentially as described by Inoue et al. (1990). *E. coli* were streaked on LB agar plates without antibiotics and cultured overnight at 37°C. A single large colony was picked with a sterile toothpick and used to inoculate 125 ml of SOB in a 1-liter Erlenmeyer flask. Flasks were incubated at 18°C with vigorous shaking (220 rpm) and the bacteria grown to an OD of 0.6 (mid-log phase). Bacteria cultures were poured into Falcon tubes and incubated on ice for 10 min and then centrifuged at 2500x g (3000 rpm) in an Eppendorf 5403 bench top centrifuge. The pellet was resuspended in 40 ml ice-cold transformation buffer (TB), incubated on ice for 10 min and centrifuged as above. The pellet was then carefully resuspended in 10 ml of TB, and DMSO added drop-wise to a final concentration of 7%. The bacterial suspension was incubated for ten minutes on ice, after which 1 ml aliquots were snap-frozen in liquid nitrogen and stored at – 80°C.

5.7.2 Transformation of competent *E. coli*

Eppendorf tubes (1.5 ml) were pre-chilled on ice and either 10 ng of purified plasmid DNA or 2 µl of ligation mixture in a total volume of 20 µl were pipetted into the tubes. Competent cells were thawed and 100 µl were dispensed into the tubes on ice. The tubes were flicked gently to mix and incubated on ice for 30 min. The cells were then heat-shocked by heating for exactly 45 s in a 42°C water bath, followed by incubation on ice for 2 min. Room temperature SOC medium (900 µl) was added to each tube on ice. The tubes were then incubated at 37°C for one hour with constant shaking. Antibiotic selective agar plates were plated with 50 to 150 µl of the transformation mixture and incubated overnight at 37°C.

5.7.3 Screening of bacterial colonies for plasmids/ recombinant plasmids

Two methods were routinely employed to identify bacteria colonies that contain the recombinant plasmids or plasmids of interest.

5.7.3.1Restriction analysis of isolated plasmids

In order to verify constructs of plasmids/inserts resulting from cloning, plasmid DNA was isolated and analysed. Independently transformed bacterial colonies were picked and grown in 5 ml LB/antibiotic overnight. Plasmid DNA was isolated (section 5.6.5) and digested (section 5.6.8) at restriction sites used for cloning of the insert DNA. The digested DNA was then analysed by agarose gel electrophoresis (section 5.6.2).

5.7.3.2 Colony PCR

Bacterial colonies were analysed if they were transformed by colony PCR. The colonies were picked using toothpick and introduced into PCR tubes with contain the PCR master mix which contain the specific primers for the gene. The PCR was carried out as mentioned in section 5.6.9 with an additional incubation at 95°C for 5 min before the PCR.

5.7.4 Bacteria cultures and long term storage of stocks

Bacteria host strains used in this study included XL 1 Blue, BL 21 (DE3) and JM109. Bacteria were streaked on LB agar plates and grown overnight at 37°C. For long-term storage of bacteria, a single colony was grown in LB overnight and sterile glycerol was added to a final concentration of 10%. Aliquots of 1ml were snap frozen in liquid nitrogen and stored at -80°C.

5.7.5 Expression and purification of protein expressed in *E. coli*

The pET expression system was used for prokaryotic expression of recombinant *A. viteae* cystatin. In this system, target genes are cloned under the control of the T7 promoter which is not recognised by *E. coli* RNA polymerase. Some *E. coli* host strains (like BL21 (DE3)) have a chromosomal copy of the T7 RNA polymerase gene under control of *lacUV5*. Transfer of recombinant pET plasmids into such hosts results in an IPTG-inducible gene expression system. The cDNA sequence of *A. viteae* cystatin with a 6 His tag at the C terminal was cloned into pET 22b vector and transformed into BL21(DE3) cells.

A single bacterial colony was picked and used to inoculate LB (15 µg/ml Kanamycin) and the culture was grown overnight at 37°C, 150 rpm. The overnight culture was diluted 1:50 in fresh 500 ml of LB medium and further grown at 37°C till an OD_{600} of 0.4. Protein expression was induced with 1mM IPTG for 4 h. Bacteria were pelleted by centrifugation at 6000 rpm for 15 min at 4°C. The bacterial pellet was suspended in 20 ml of 1X PBS pH 7.4, with 0.1% Triton and 100 µl/ml lysozyme and kept on ice for 30 min. It was sonicated for 3 min and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was passed through an equilibrated Ni-NTA column (1 ml). The charged column was washed with 1X PBS with pH ranging from 7.4 to 5. The protein bound to the Ni-NTA was eluted in fractions of 500 µl with PBS, pH 3. The eluted protein was dialysed overnight against 1X PBS pH 7.4. The various fractions of washing and elution were analysed by SDS-PAGE and Western blot (section 4.4.2). The pure protein was stored at -20°C to be used for immunisations and ELISA

5.8 Microscopy

Light fluorescence and fluorescence microscopy were done using a Zeiss Axioplan fluorescence microscope with a blue excitation filter set (Number 487909). For detection of EGFP (enhanced green fluorescent protein) fluorescence in mammalian cells, the transfected COS7 and HeLa cells were suspended in PBS and mounted on slides. The detection was recorded on a digital camera mounted onto the microscope. For the detection of GFP in *C. elegans* and FITC labelling in *A. viteae*, the samples were mounted on slides and observed using a confocal laser-scanning microscope (Lieca TCS SP) with an argon/krypton gas laser. The gas laser was set to 50 – 60% and the detection of GFP was done at 488 nm.

5.9 Liposomal encapsulation of DNA

Plasmid DNA was encapsulated in liposomes to enhance the transfection of host cells by DNA when immunized. According to the protocol from Gregoriadis G et. al., plasmid DNA (up to 2 mg) encoding *A. viteae* cystatin was incorporated by the dehydration–rehydration method into liposomes. The liposomes composed of 32 µmol egg phosphatidylcholine (PC), 32 µmol Cholesterol and 8 µmol 1,2-diodeoyl-3-(trimethylammonium) propane (DOTAP) (cationic liposomes). The method entails

mixing of small unilamellar vesicles (SUV) with the DNA followed by dehydration and rehydration. Dehydration of the DNA–SUV complexes and subsequent rehydration, generated submicron size liposomes incorporating most of the DNA in a fashion that prevents DNA displacement through anion competition. DNA is entrapped within the aqueous compartments, in between bilayers, bound to the cationic charges.

For the synthesis of SUV, PC, Cholesterol and DOTAP were dissolved in chloroform to a final volume of 5 ml in a round bottom flask and the solvent was removed using a rota evaporator to obtain a thin film of the lipids. Purified plasmid DNA (up to 2 mg) in 2 ml of HPLC water was added to the lipid film and flushed with N_2 to remove air. The flask containing the synthesis mixture was incubated at 50°C for 30 min with vigorous vortexing every 5 min yielding large unilamellar vesicles (LUV). The synthesis mixture which contains LUV was transferred in to a flacon tube and incubated at 50°C for 30 min. It was sonicated for 5 min to obtain SUV and was stabilised by incubation at 50°C for 30 min and was snap frozen in liquid N_2. The SUV and plasmid DNA was dehydrated in a freeze dehydrator for overnight. It was rehydrated gradually at 45°C by adding water in amounts of 100 μl to make up the volume to 1 ml and centrifuged at 4°C at 25,000 rpm. The pellet was washed with 1X PBS twice and resuspended in 1 ml of sterile water. The same procedure was followed to obtain liposomal encapsulated negative control plasmid DNA. The encapsulation of the DNA was proofed by agarose gel electrophoresis. The encapsulated DNA was mixed with 1 μl of 6X agarose loading buffer with or without 1% SDS which neutralizes the cationic liposomes and releases the encapsulated DNA. The amount of DNA encapsulated is calculated based on the intensity of the released plasmid as compared to the marker lane and accordingly was used for immunization studies.

5.10 Expression and purification of protein expressed in C. elegans

Large liquid cultures (200 ml) of *C. elegans* were grown as mentioned in section 5.3.1 and bacteria removed as mentioned in section 5.3.2. Transgenic worms which expressed *A. viteae* genes under the heat shock promoter were incubated at 33°C for 3 h and pelleted down. The worms were sonicated in 5 ml of 1X PBS containing 0.1% Triton, pH 7.4. The supernatants of the lysates were charged on Ni-NTA column and washed as mentioned in section 4.2.5. The elution was done with

PBS (pH 3.0) and the fractions were loaded on SDS-PAGE and proofed by western blot.

5.11 Immunisation experiments

The protective potential of *A. viteae* cystatin was evaluated in the *Meriones/A. viteae* natural host-parasite system. Eight to ten weeks old *Meriones* were anaesthesized with ketamin:xylazin:normal saline (1:1:8) and immunised with *A. viteae* cystatin as naked plasmid DNA vaccine or plasmid DNA encapsulated in liposome or as protein. For DNA immunization studies, 50 μg of plasmid per animal was used intramuscular in the thigh muscles. For protein immunization, 25 μg of protein per animal was used subcutaneously. The animals were immunised three times with two weeks intervals, after which they were challenged with 70 freshly isolated L3s by injecting subcutaneously in the neck region. Microfilarial load was verified at weeks 8 and 11 post infection (p.i.), and the animals were dissected at week 11 p.i. for isolation of adult worms and determination of protective potential. Different adjuvants were used for immunisation, depending on the immune reaction desired: Alum was used for humoral-mediated immune reactions, STP was used for cell-mediated immune responses and AdjuPhos was used with DNA vaccine.

Statistical analyses

The worms recovered from immunised and non-immunised *Meriones* in immunisation experiments were compared using the Mann-Whitney U-test at the 95% significance level ($p<0.05$).

5.12 RNA interference experiments

5.12.1RNAi by soaking with dsRNA

5.12.1.1 Synthesis of template for double stranded RNA

The templates required for the synthesis of dsRNA of cystatin, chitinase and tropomyosin were amplified by PCR using gene specific primers with T7 promoter sequences at the 5' end of the primers. For cystatin, the full length sequence of 474 bp of the cDNA was PCR amplified. For chitinase, the full length of 1500 bp and the region corresponding from 964 to 1464 of the cDNA sequence was amplified. Also, the DNA sequence of the bacterial maltose binding protein (NEB) was amplified which was used for the synthesis of the negative control dsRNA. The PCR amplified products were purified by phenol-chloroform and precipitated by ethanol and NaCl as mentioned in section 5.7.4. The DNA pellets were dissolved in nuclease free water and used for the synthesis of dsRNA

5.12.1.2 Synthesis of double-stranded RNA (dsRNA)

Double stranded RNA was synthesised using the MegaScript RNAi Kit (Ambion) according to the manufacturer's instructions. T7 RNA polymerase synthesises RNA from the DNA template and the single stranded RNA anneal together to form dsRNA. The reagents were pipetted according to the following scheme.

Table 5.3 Pipetting scheme for synthesis of dsRNA

Component	Amount
Nuclease free water	Up to 20 μl
DNA template	1 -2 μg
10X Reaction buffer	2 μl
ATP	2 μl
CTP	2 μl
GTP	2 μl
UTP	2 μl
T7 enzyme mix	2 μl

The reaction mix was incubated at 37°C for 3 h and then heated to 75°C and cooled to room temperature to anneal the complementary single strands of RNA. The dsRNA was treated with DNase and RNase to remove the DNA template and single stranded RNA. The dsRNA of cystatin (AvCys dsRNA), of chitinase full length (AvChifl dsRNA), of 3' chitinase (AvChi3' dsRNA), of tropomyosin (AvTropo dsRNA)

and of bacterial maltose binding protein (Mal dsRNA) were purified using the Megascript RNAi Kit columns and the concentration was measured using Nanodrop Spectrophotometer. The purified dsRNA was used for further RNAi experiments.

5.12.1.3 Treatment of *A. viteae* with dsRNA by soaking

A. viteae L3 worms isolated from the ticks were washed 3 times with cRPMI but without serum and aliquoted into 96 well plate with 100 L3 per 50 µl of cRPMI with serum per well. Double stranded RNA (Cys dsRNA or Chifl dsRNA or Chi3' dsRNA or Tropo dsRNA or Mal dsRNA) was added to the worms such that the molar concentration was 75.5 pmol / 100 µl. They were incubated at 37°C for 16 h and frozen for RNA isolation for quantification of transcripts. For observing the effect on moulting of L3 to L4, the L3 worms treated with dsRNA were used to infect *Meriones* and the late L3 stage was isolated after 5 days as mentioned in section 5.1.3.3.

A. viteae adult worms were isolated from *Meriones*, washed in cRPMI but without serum and checked for their integrity under a binocular microscope. Female worms in groups of 5 or males in groups of 10 in dialysis tubings were treated with dsRNA at a concentration of 75.5 pmol in 100 µl of RPMI. The negative controls had worms without dsRNA or with dsRNA of the bacterial maltose binding protein (Mal dsRNA). The dialysis tubings containing the worms were immersed into 50 ml tubes containing RPMI and incubated at 37°C for 16h. For isolation of RNA, the worms were snap frozen in liquid N_2. To observe the phenotype, the worms were transferred to 5 ml of fresh complete RPMI and maintained for another 5 days at 37°C with change of medium every 24 h.

5.12.2 RNAi by small interfering RNA (siRNA) by soaking

To analyse if siRNAs could induce RNAi in *A. viteae*, the worms were treated to siRNAs of cystatin by soaking. The siRNA's were designed using the online siRNA target finder (www.ambion.com/techlib/misc/siRNA_finder). The program scan for AA dinucleotide sequences beginning with the AUG start codon of entered transcript and record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. that siRNAs with 3' overhanging UU dinucleotides are the most effective. This is

also compatible with using RNA pol III to transcribe hairpin siRNAs because RNA pol III terminates transcription at 4-6 nucleotide poly(T) tracts creating RNA molecules with a short poly(U) tail. The siRNAs were chosen such that they have about 30-50% GC content and also they did not have stretches of >4T or A's. This was because poly (T) tract acts as a termination signal for RNA pol III. The siRNA's designed for *A. viteae* cystatin, Cysi1, Cysi2 and Cysi3, were compared against nematode database to determine if there was any homology to any other sequences. The siRNA's designed for *A. viteae* cystatin are shown in figure 5.2. The double stranded siRNA's were custom synthesised by Ambion and dissolved in nuclease free water to obtain a concentration of 50 μM.

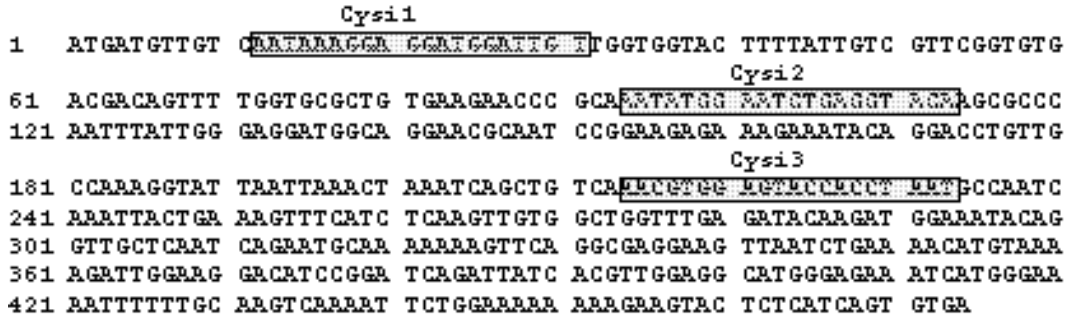


Figure 5.2 The siRNA's designed for cystatin

The siRNA's, Cysi1, Cysi2 and Cysi3, are boxed in grey and span 21 nucleotides in length.

A. viteae L3 worms were soaked with Cysi1, Cysi2 and Cysi3 to observe if they were amenable to RNAi by siRNA's. About 100 L3 *A. viteae* worms were soaked in each siRNA at a concentration of 2 μM in incomplete RPMI (without FCS). Totally about 300 worms were used for each siRNA. The negative control was worms in RPMI. They were incubated at 37°C for 16 h and the RNA was isolated for quantification of cystatin transcripts by real-time PCR.

5.12.3 RNAi in *A. viteae* L3 larvae by the electroporation method

Studies with parasitic nematodes have suggested that electroporation with dsRNA or siRNA could induce a better RNAi effect than soaking. Therefore, *A. viteae* L3 worms were electroporated with AvCys dsRNA or AvChi dsRNA or AvTropo dsRNA or Mal dsRNA, and with siRNA's of cystatin. *A. viteae* L3 larvae were suspended in trehalose electroporation buffer (100 worms in 100 μl) containing

75.5pmol / 100μl of either AvCys dsRNA, AvChifl dsRNA, AvChi3' dsRNA or Mal dsRNA. Also, *A. viteae* L3 larvae were electroporated with Cysi1 or Cysi2 or Cysi3 at concentrations of 2 μM and 1 μM in electroporation buffer. The electroporations were done in 2 mm cuvettes. The different conditions used for the electroporation of L3 worms are summarized in table 5.4. After the electroporation, 100 μl of warm RPMI was added to the cuvettes and the worms were allowed to recover for 5 min. The worms were transferred to 96 well plates and incubated further at 37°C for 16 h, after which the worms were snap frozen in liquid nitrogen for the isolation of RNA.

Table 5.4 Various conditions used for the electroporation of *A. viteae* L3 worms

dsRNA / siRNA	Concentration	Voltage	Pulse length
Cys dsRNA Chifl dsRNA / Chi3' dsRNA Tropo dsRNA Mal dsRNA	75.5 pmol/100μl	100 V	100 μs
		150 V	150 μs
		200 V	100 μs
Cysi 1 Cysi 2 Cysi 3	2 μM	100 V	100 μs
		150 V	150 μs
		200 V	100 μs

5.13 Real-time PCR

The transcript amounts of cystatin, chitinase and tropomyosin in *A. viteae* L3 and adult worms after RNAi were quantified by real-time PCR. It was also used to determine the transcript amounts of cystatin and chitinase during the different stages of *A. viteae*. TaqMan MGB (Minor Groove Binding) probe-based 5' nuclease chemistry was used for the real-time PCRs. This involves the use of forward and reverse primers to amplify sequences about 70 bp to 100 bp and a probe which is labelled and specific to the amplicon. The fluorogenic probe enables the detection of a specific PCR product as it accumulates during PCR cycles. The TaqMan MGB probes for cystatin, chitinase and tropomyosin were labeled with 6-carboxyfluorescein (FAM) at the 5' end and a non-fluorescent quencher at the 3' end. It also has a minor groove binder at the 3' end which binds to the minor groove of the helically twisted double stranded DNA [218, 219]. This increases the melting temperature of the probe and therefore the higher sensitivity for the real-time PCR. During the annealing phase, the probe specifically annealed to the target sequence.

The forward and reverse primers anneal to the complementary sequences and were extended (Fig. 5.3). Due to the 5' exonuclease activity of DNA polymerase, the hybridized probe was hydrolyzed leading to the separation of the reporter moiety from the quencher moiety and the generation of a fluorescent signal. The signal was detected by ABI 7300 real-time PCR system (Applied Biosystems Inc. California, USA).

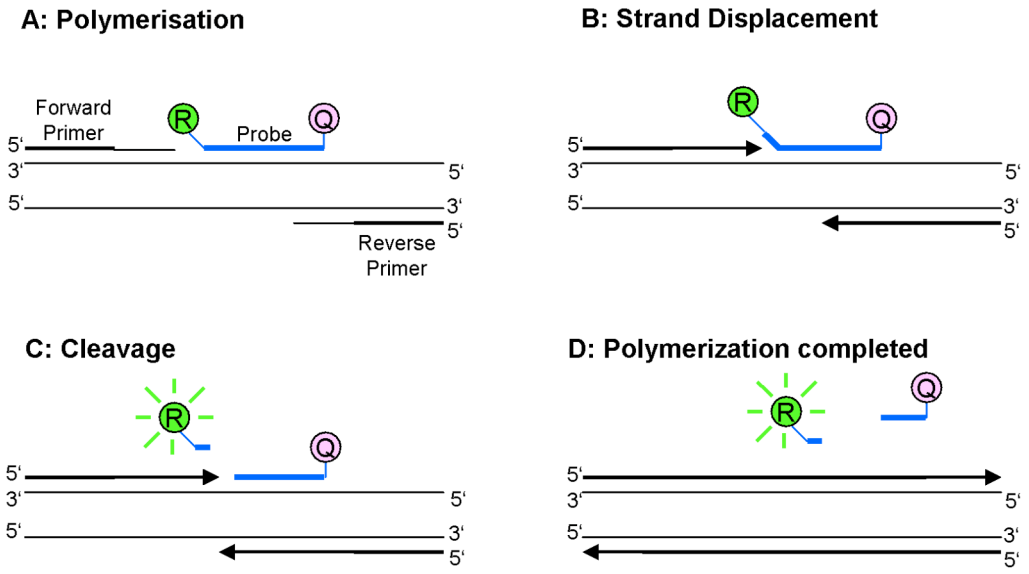


Figure 5.3 TaqMan probe-based chemistry used for Real-Time PCR.

A: During polymerisation the forward and reverse primers and the probe with the reporter (R) at the 5' end and quencher (Q) at the 3' end bind to the template strand.

B: The strand is displaced but as long as both the dye and quencher are attached to the probe, reporter dye emission is quenched.

C: During each extension the DNA polymerase (AmpliTaq Gold ®) cleaves the reporter dye from the probe.

D: After being separated from the quencher, the reporter dye emits its characteristic fluorescence.

5.13.1 Designing of primers

The primers for real-time PCR for the genes *A. viteae* chitinase, cystatin and tropomyosin were designed at the exon-exon juctions in the cDNA sequences. The guidelines used for the designing of the primers and probes are: a) The amplicon should be short about 100 bp and should span one or more introns to avoid genomic DNA contamination, b) The primers and probe should have a G/C content of 30 to 80%, c) The probes should have a T_m of 68 to 70°C and have no G on the 5' end, d) The primers should have a T_m of 58 to 60°C. The primers and the probes of cystatin,

chitinase and tropomyosin of *A. viteae* were custom synthesised and supplied by Applied Biosystems Inc. at a 20X concentration. The primers and probes used for real-time PCR of cystatin, chitinase and tropomyosin are shown in table 5.5.

Table 5.5 PCR primers and probes used for real- time PCR.

Gene	Primer and. Probe sequences	Position
Cystatin	Forward: AGGCGAGGAAGTTAATCTGAAAACA Reverse: CTCCCATGCCTCCAACGT Probe : ATCCGGATCAGATTATC	330 – 354 391 – 408 374 – 390
Chitinase	Forward: GGAGAGAGAAACATCCGGAACTG: Reverse: CTTTCTCGGCCCAATAATTTGCT Probe: TCTGTAATACCGAATTTGC	717 – 739 762 – 784 743 – 761
Tropomyosin	Forward: GGAAAAAGCAACTCATACAGCTGATG Reverse: TGTTAGCACGTTCTTCATCTTGGAA Probe : CCGACCGCGTTCGCA	339 – 364 403 – 427 368 – 382

5.13.2 cDNA synthesis for real-time PCR

Total RNA from worms were isolated as mentioned in section 5.6.5. The cDNA for quantitative PCR (qPCR) was synthesised using the TaqMan cDNA synthesis kit (Applied Biosystems Inc. California, USA). The reagents for reverse transcription were pipetted according to the following scheme.

Component	Amount
Nuclease free water	Up to 20 ul
Total RNA	10 ng to 10 μg
10 X Reaction Buffer	2 μl
Oligo dT	1 μl
2.5 mM dNTP	4 μl
25 mM $MgCl_2$	4.4 μl
RNase Inhibitor	0.4 μl
MultiScribe Reverse Transcriptase (50 U/μll)	0.5 μl

The reaction mix was incubated at 25°C for 5 min followed by incubation at 48°C for 30 min and inactivation of the enzymes at 95°C for 10 min. The synthesised cDNA was used in the quantitative PCRs.

5.13.3 Quantitative PCR (Real-Time PCR)

A PCR Master Mix was prepared according the following scheme, the volume of which depended on the number of reactions to be set up.

Reaction Component	Final Concentration
TaqMan Universal PCR Master Mix (2X)	1X
Primer Probe Mix (20 X)	1X
cDNA sample	10 to 100 ng
Nuclease free water	Up to 25 μl

A reaction mixture of 25 μl for the target genes and the endogenous control were pipetted singleplex into an Optical 96-Well Plate. The PCR was run on the thermal cycler. The conditions for the thermal cycler were 95°C for 10 min for polymerase activation and 40 cycles of 95°C for 15 s and anneal/extend for 1 min at 60°C. The data were analysed using the SDS-software for the instrument. The results of qPCR are displayed as amplification plots for each gene. The baseline and the threshold are set such that they are within the exponential phase of the amplification curves. The SDS software calculates the C_t values, which is defined as a fractional cycle at which the fluorescence of the sample passes through a threshold.

Initially, serial dilutions of cDNA (1:10, 1:100, 1:1000, 1:10,000) were tested and plotted against the threshold cycles received by fluorescence measurements were during each annealing step, in order to obtain a standard curve. In subsequent experiments only those dilutions that had Ct values within the linear range of the standard curve were used. Primer efficiencies (E) were calculated from the slope of the standard curve (E=10 (-1/10)) and found to be close to 2. This ensures that PCR products have been doubled.

Threshold cycles determined for the target gene as well as for the endogenous control gene are further used to calculate relative amounts of the two genes in any given samples. The relative quantification was calculated using the comparative C_t method according to the formula described below.

Relative quantification = $2^{-\delta\delta Ct}$

where $\delta\delta C_t$ = (δC_t of target sample) – (δC_t of reference sample)

where δC_t = (C_t of target gene) – (C_t of endogenous gene)

where C_t is the cycle number in the PCR where threshold amount of amplicons are made.

6 MATERIALS

6.1 *Caenorhabditis elegans* strains

Wild type-N2 strain	Kind gift from Bernhard Nocht Institute, Hamburg
GE-42 (pha-1 mutants)	Caenorhabditis Genetics Center, Minneapolis

6.2 Laboratory equipment

Nanodrop	PeqLab, Germany--
Electrophoresis apparatus	Pharmacia Biotech, Freiburg AGS, Heidelberg
Cryocut E Reichert-Jung	Leica, Solms
Electronic precision weighing instrument BP 3100 S	Sartorius, Göttingen
ELISA-Reader MRX and washing instrument	Dynatech MRW Laboratories, Denkendorf
Incubators	
Warmincubator	Memmert, Schwabach
Shaking incubator	New Brunswick Scientific, Netherland
Magnetic stirrer	IKA Labortechnik, Staufen
Microscope	
Leica DM/R	Leica, Solms
Zeiss Axioplan	Zeiss, Oberkochen
Leica TCS NT	Leica, Solms
Confocal laser-scanning microscope (TCS SP)	Leica, Solma
pH-Meter	WTW, Weilheim
Precision pipettes	Eppendorf, Hamburg
Sterile work bench	Heraeus Christ, Zürich
Thermoblock	
MJ Research PTC-200	BIOzym, Hessisch Oldendorf
PCR gradient cycler	Eppendorf, Hamburg
7300 Real-Time PCR System	Applied Biosystems, Darmstadt
Ultraschall-Desintegrator, Sonifier W-250	HTU, Schwäbisch Gmünd
Video-Dokumentations instrument E.A.S.Y. RH	Herolab, Wiesloch
Waterbath DC3	Haake, Karlsruhe
Centrifuges	
Cooling centrifuge 4804 R	Eppendorf, Hamburg
Table centrifuge 5415 C	Eppendorf, Hamburg
Large volume cool centrifuge J2-HS	Beckmann, München
Gamma-Irradiator Biobeam 2000	STS, Braunschweig
Video documentation apparatus E.A.S.Y.RH	Herolab, Wiesloch
Bandelin Sonoplus HD 200 sonicator	Berlin

6.3 Primers

The primers were synthesised by TIB-MOLBIOL, Berlin. The restriction enzyme sequences included in the primers are within brackets.

6.3.1 Primers for Cystatin

49CysF (*Nhe* I)	5′ TATTCAGCTAGCATGATGTTGTCAATAAAG 3′
49CysR (*Kpn* I)	5′ TATTCAGGTACCTCAATGGTGATGGTGATGGTGATGCACTGATGAGAGTAC 3′
103CysF (*Nhe* I)	5′ TATTCAGCTAGCATGCACCATCACCATCACCATATGATGTTGTCAATGAAG 3′
103CysR (*Sma* I)	5′ TCCCCCGGGTCATCACAATCGACTTTA 3′
prCysR (*Kpn* I)	5′ TATCCGCTACCATCGTCGTTAGCTTTGTTT 3′

prCys95F (*Hind* III)	5′ CCCAAGCTTTAACCCTCACTAAAGGGA 3′
prCys95R (*Pst* I)	5′ AACTGCAGATTGCGTTCCTGCCATCC 3′
pCDCysF (*Bam*HI)	5′ CGCGGATCCATGATGTTGTCAATAAAG 3’
pCDCysR (*Xba* I)	5′ TGCTCTAGATCACACTGATGAGAGTACT 3′
CysGfpF (*Hind* III)	5′ CCCAAGCTTATGATGTTGTCAATAAAG 3′
CysGfpR (*Pst* I)	5′ AAAACTGCAGCACTGATGAGAGTACT 3′
CysT7F	5′ TAATACGACTCACTATAGGGATGATGTTGTCAATAAAG 3
CysT7R	5′ TAATACGACTCACTATAGGGTCACACTGATGAGAGTACT 3′
T7 primer	5′ TAATACGACTCACTATAGGG 3′

6.3.2 Primers for Chitinase

prChiGfpF (*Bgl* II)	5′ CGAGATCTCTTTGGTAATTAAACCAT 3′
prChiGfpR (*Sac* I)	5′ CGAGCTCATTTGCAACAGTAATTATA 3′
ChiflT7F	5′ TAATACGACTCACTATAGGG
Chi3’T7F	5′ TAATACGACTCACTATAGGGGCAAGGAAACGGTGGATAA 3′
Chi3’T7R	5′ TAATACGACTCACTATAGGGTGTGATGCGGAAATAAACC 3′

6.3.3 Primers and TaqMan Probes used for Real-Time PCR

Cystatin

Forward	5′ AGGCGAGGAAGTTAATCTGAAAACA 3′
Reverse	5′ CTCCCATGCCTCCAACGT 3′
Probe	5′ ATCCGGATCAGATTATC 3′

Chitinase

Forward	5′ GGAGAGAGAAACATCCGGAACTG 3′
Reverse	5′ CTTTCTCGGCCCAATAATTTGCT 3′
Probe	5′ TCTGTAATACCGAATTTGC 3′

Tropomyosin

Forward	5′ GGAAAAAGCAACTCATACAGCTGATG 3′
Reverse	5′ TGTTAGCACGTTCTTCATCTTGGAA 3′
Probe	5′ CCGACCGCGTTCGCA 3′

6.4 Vectors and Bacterial strains

6.4.1 Vectors

pEGFP-N1	Clontech, BD Biosciences, Heidelberg
pcDNA 3	Invitrogen
pSL-1180	Amersham Biosciences
pGEMT	Promega, Mannhiem
pPD 49.83	Caenorhabditis Genetics Centre
pPD 103.05	Caenorhabditis Genetics Centre
pPD 95.77	Caenorhabditis Genetics Centre

6.4.2 *E. coli* strains

E. coli BL21 (DE3)	Stratagene, Heidelberg
E. coli DH5 α	Promega, Heidelberg

E. coli JM109	Stratagene, Heidelberg
E. coli XL1 Blue	Clontech BD Biosciences, Heidelberg

6.5 Consumables

Biodyne A Transfer Membranes	Pall, Portsmouth, England
Tissue Tek® Cryomold® Biopsy	Miles Inc., Elkhart, USA
Dialysis tubings	Serva, Heidelberg
Disposable pipettes (10 and 25 ml)	Costar, Bodenheim
Cryotubes	Greiner, Nürtingen
Microtiterplates (Flat bottom)	Corning Life Science, Schiphol, Niederlande
Nitrocellulose-Membrane	Schleicher & Schuell, Dassel
Nitrocellulose Filter Protran BA 83	Schleicher & Schuell, Dassel
Nunc Immuno™-Plates, 96 wells	Nunc, Wiesbaden
Microscopic slides, SuperFrost®*/Plus	Menzel-Gläser, Braunschweig
Petriplates, ø 14 cm und 145 cm	Greiner, Nürtingen
Pipette tips	Sarstedt, Nürnbrecht Greiner Nürtingen
Pipetten tips, RNAse frei	Roth, Karlsruhe
Polypropylene centrifuge tubes (15 und 50 ml)	Greiner Nürtingen
S-Monovette®	Sarstedt, Nümbrecht
Sterile filters	Schleicher & Schuell, Dassel
Whatman-Paper	Schleicher & Schuell, Dassel
Cell culture flasks	Biochrom, Berlin
Cell scraper	Biochrom, Berlin
Femtotips Microinjection needles	Eppendorf, Hamburg
Femtojet Microinjector	Eppendorf, Hamburg
Biolistic PDS 1000/He system	Biorad, Germany
Gold microcarriers	Biorad, Germany
Macrocarriers	Biorad, Germany
Microcapillaries	Roth, Karlsruhe
Electroporation cuvettes	Biorad
Fuchs-Rosenthal chamber	Roth, Karlsruhe
Cryovials	Cellstar, Frickenhausen
Microhaematocrit capillaries	Roth, Karlsruhe

6.6 Reagents

Acrylamide-Bisacrylamide-Solution, Rotiphorese Gel 30	Roth, Karlsruhe
Acetic acid	AppliChem, Darmstadt
Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Ammoniumpersulfate (APS)	Serva, Heidelberg
Antibiotics	AppliChem, Darmstadt
Bromophenol blue	Merck, Darmstadt
5-Bromo-4-chloro-3-indolylphosphate disodium salt (BCIP)	Roth, Karlsruhe
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)	AppliChem, Darmstadt
Bovine serum albumin (BSA)	Sigma, Deisenhofen
Benzoyl-DL-Arg-p-nitroamilide (BAPNA)	Sigma, Deisenhofen
Chlornapthol	AppliChem, Darmstadt
Cholesterol	Sigma, Deisenhofen
Chloroform	Merck, Darmstadt
Citric acid	Roth, Karlsruhe
CSPD	Roche, Mannheim
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe
N,N-Dimethylformamide (DMF)	AppliChem, Darmstadt
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe

1,2-diodeoyl 3 (trimethylammonium) propane	Avanti Lipids, Hamburg
dNTPs	Rapidozym, Berlin
Ethylenedinitrotetraacetic acid (EDTA)	AppliChem, Darmstadt
Egg Phosphaditylcholine	Sigma, Deisenhofen
Ethanol pure	AppliChem, Darmstadt
Ethidiumbromide	AppliChem, Darmstadt
Fetal calf serum	Biochrom, Berlin
Fugene	Roche, Mannhiem
Glucose	AppliChem, Darmstadt
L-Glutamine	Biochrom, Berlin
Glutaraldehyde	Sigma, Steinheim
Glycine	AppliChem, Darmstadt
Urea	AppliChem, Darmstadt
Yeast extract	AppliChem, Darmstadt
Isopropyl-β-D-thiogalactopyranoside (IPTG)	AppliChem, Darmstadt
Lysozyme	AppliChem, Darmstadt
Lipofectamin	Life Technologies, Karlsruhe
Magnesiumchloride	AppliChem, Darmstadt
Malic acid	Roth, Karlsruhe
Maltose	AppliChem, Darmstadt
MEM-EARLE Medium	Biochrom, Berlin
Methanol, analytical	AppliChem, Darmstadt
2 C 180 Methylene blue Unna	ChromaGesellschaft, Münster
Monohydrate-Citric acid	Roth, Karlsruhe
3-Morpholinopropanesulfonic acids (MOPS)	AppliChem, Darmstadt
Mowiol® 40-88	Sigma, Steinheim
p-Nitrobluetetrazoliumchloride (NBT)	Roth, Karlsruhe
p-Nitrophenyl-N-Acetyl-ß-D-Glucosamine	Sigma, Steinheim
Ni-NTA-Matrix	QIAGEN, Hilden
Papain	Sigma, Steinheim
Paraformaldehyde (PFA)	Merck, Darmstadt
Penicillin/Streptomycin (10000 U/10000 μg/ml)	Biochrom, Berlin
Phenol	Sigma, Steinheim
Phenol/Chloroform/Isoamylalcohol	Roth, Karlsruhe
Phenylmethylsulfonylfluoride (PMSF)	Fluka, Steinheim
Phosphatase Substrate tablets	Sigma, Steinheim
RPMI-Medium	Biochrom, Berlin
D(+)-Saccharose	AppliChem, Darmstadt
Sodium acetate	AppliChem, Darmstadt
Sodium borohydrate	Roth, Karlsruhe
Sodium chloride	AppliChem, Darmstadt
Sodium citrate	Roth, Karlsruhe
di-Sodium hydrogen phosphate	AppliChem, Darmstadt
Sodium meta peroxide	AppliChem, Darmstadt
Sodium hypochloride	Roth, Karlsruhe
Sodiumdodecyl sulfate(SDS)	AppliChem, Darmstadt
Squalane	Roth, Karlsruhe
TEMED	Roth, Karlsruhe
Tissue Tek®	Sakura, Zoeterwoude
3,3´,5,5´-Tetramethylbenzidine (TMB) tablets	Sigma, Steinheim
Tris Base	AppliChem, Darmstadt
Triton X-100	Serva, Heidelberg
Trypton	AppliChem, Darmstadt
Trypsin-EDTA	Biochrom, Berlin
Tween 20	AppliChem, Darmstadt
Uranyl Acetate	Merck, Darmstadt
Hydrogen peroxide (H_2O_2)	Roth, Karlsruhe

6.7 Commercial Kits

BCA Protein Assay Kit	Pierce, USA
Gel drying-Kit	Promega, Heidelberg
NucleoSpin® Mini Plasmid Kit	Macherey & Nagel, Düren
NucleoBond® Midi Plasmid Kit	Macherey & Nagel, Düren
NucleoSpin® Extraction Kit	Macherey & Nagel, Düren
NucleoTrap® Gel Extraction Kit	Macherey & Nagel, Düren
pGEM®-T Easy Vector System I	Promega, Mannheim
RNeasy® Mini Kit	QIAGEN, Hilden
QIAshredder spin columns	QIAGEN, Hilden
Megascript RNAi Kit	Ambion, Cambridgeshire, UK
siRNA's Kit	Ambion, Cambridgeshire, UK
TaqMan primer and probes	Applied Biosystems, Darmstadt
TaqMan cDNA synthesis Kit	Applied Biosystems, Darmstadt
TaqMan PCR master mix	Applied Biosystems, Darmstadt
prestained Protein Marker, Broad Range	New England Biolabs, Schwalbach
1 kb DNA Ladder	Rapidozym, Berlin

6.8 Enzymes

DNA-Restriktion enzymes	New England Biolabs, Schwalbach
Lysozyme	AppliChem, Darmstadt
Superscript III Reverse Transcriptase	
Proteinase K	Sigma, Steinheim
Taq-Polymerase	Rapidozym, Berlin
Phusion polymerase	
T4 DNA-Ligase	New England Biolabs, Schwalbach

6.9 Solutions, Medium and buffers

6.9.1 Agarose gel electrophoresis buffers

6 x Agarose-Loading buffer	40% D(+)-Saccharose 0.005% Bromphenolblue
Ethidiumbromide-solution	1% in sterile. H_2O
10 x FA-Gel-buffer	200 mM MOPS 50 mM Sodium acetate 10 mM EDTA in RNase-free H_2O pH 7
1 x FA-Gel running buffer	1 x FA Gel-buffer 0.74% Formaldehyde in RNase-free. H_2O
5 x RNA Loading buffer	0.16% ges. Bromphenol blue solution 4 mM EDTA, pH 8 2.7% Formaldehyde 20% Glycerol 30.1% Formamide 4 x FA Gel buffer in RNase-free H_2O
TAE-buffer	40 mM Tris-HCl 1 mM EDTA 0.11% Acetic säure pH 8

6.9.2 Bacterial and *Caenorhabditis* culture medium

IPTG stock solution	1 M IPTG in dest. H_2O
LB-Medium	0.5% NaCl 1% Trypton 0.5% Yeast extract in H_2O, pH 7.5, autoclaved
LB-Agar	LB-Medium 1.5% Agar autoclaved
SOB-Medium	0.05% NaCl 0.5% Yeast extract 2% Tryptone 2,5 mM KCl 10 mM $MgCl_2$ Autoclaved
SOC-Medium	SOB-Medium 20 mM Glucose, sterile filtered
TB-Buffer	10 mM PIPES 15 mM $CaCl_2$ 250 mM KCl pH 6.7 55 mM $MgCl_2$ Sterile filtered
X-Gal	2% in DMF
1 M Potassium citrate pH 6.0	2% citric acid monohydrate 30% Tri-potassium citrate monohydrate H_2O, autoclave
Nematode Growth medium	0.3% NaCl 1.7% Agar 0.25% Peptone In H_20 and autoclave After cooling to 55°C add 1mM $CaCl_2$ 0.0005% Cholesterol 1mM $MgSO_4$ 25mM KPO_4
Trace metals solution	0.18% disodium EDTA 0.07% $FeSO_4$ $7H_2O$ 0.02% $MnCl_2.4H_2O$ 0.03% $ZnSO_4.7H_2O$ 0.003% $CuSO_4$ $5H_2O$
S-Basal	0.58% NaCl 0.1% K_2HPO_4 0.6% KH_2PO_4 0.0005% Cholesterol In H_2O
S-medium	1 L of S-Basal 10mM Potassium Citrate, pH 6 1% Trace Metal solution 3mM $CaCl_2$ 3mM $MgSO_4$

	Filter sterile
M9 buffer	0.3% K_2HPO_4 0.6% Na2HPO4 1mM $MgSO_4$
10X Microinjection buffer	200mM KPO_4 30mM K citrate 20% polyethylene glycol (PEG) 6000 pH 7.5
Trehalose electroporation buffer	272mM Trehalose 7mM KH_2PO_4 1mM $MgSO_4$

6.9.3 Antibiotics

Ampicillin	100 mg/ml in. H_2O in cultures: 1/1000
Chloramphenicol	34 mg/ml in. H_2O in culture: 1/1000
Kanamycin	25 mg/ml in H_2O in culture 1.5/1000
Tetracyclin	20 mg/ml in Ethanol in culture: 1.5/1000

6.10 Protein and Immunochemistry

6.10.1 SDS-PAGE

APS-Stock solution	10% APS in H_2O
Coomassie-Staining solution	10% Acetic acid 20% Ethanol 0.2% PhastGel®BlueR
Coomassie-Destaining solution	10% Acetic acid 20% Ethanol
Running buffer	190 mM Glycine 25 mM Tris-HCl 0.1% SDS
Solution L	1.5 M Tris-HCl 0.4% SDS pH 8.4
Solution M	0.5 M Tris-HCl 0.4% SDS pH 6.8
2 x Loading buffer	10% Glycerin 5% β-Mercaptoethanol 3% SDS 100 mM Tris-HCl 0.02% Bromphenolblau

6.10.2 Solutions for SDS-Polyacrylamide gel

	6% (Stacking gel)	**10% (Resolving gel)**	**12% (Resolving gel)**
Acrylamide (30%)	0.375 ml	1.65 ml	2 ml
H_2O	1.5 ml	2.05 ml	1.75 ml
Solution L	-	1.25 ml	1.25 ml

Soluition M	0.625 ml	-	-
TEMED	2.5 µl	5 µl	5 µl
APS (10%)	25 µl	50 µl	50 µl

6.10.2 Western Blot

AP-Detection buffer	100 mM Tris-HCl 100 mM NaCl 5 mM $MgCl_2$ pH 9.5
BCIP-Stock solution	5% in DMF (100%)
NBT-Stock solution	5% in DMF (70%)
PBS	171 mM NaCl 3.4 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 pH 7.4
Transfer buffer	48 mM Tris-HCl 39 mM Glycine 0.037% SDS 20% Methanol
TBS-T	TBS 0.05% Tween 20 pH 7.4

6.10.4 Immunostaining

Wash solution	0.1 M Tris-HCl pH 7.5 and PBS/ 0.05% Tween
Blocking solution	10% FCS in PBS pH 7.4
Fixing solution	2.5% Paraformaldehyde in PBS pH 7.3 and 4% formaldeyde in PBS
Mowiol mounting medium	7 g Glycerol 2.4 g Mowiol 6 ml H_2O Mix at RT for 2 h 12 ml 0.2 M Tris-HCl pH 8.5 1 h at 50 °C

6.10.5 Protease inhibitors

Solution I	100 mM PMSF in Isopropanol
Solution II	100 mM EDTA (Titriplex III) 100 mM ε-Aminocapric acid 100 mM Benzamidine

6.10.6 Ni-NTA-Affinity chromatography

Lysis buffer	PBS 0.1 % Triton X

	Lysozyme pH 8
Wash buffers	PBS pH 6.3 PBS pH 5.9
Elution buffer	PBS pH 4.1

6.10.7 ELISA

Coating buffer	13 mM Na_2CO_3 35 mM $NaHCO_3$ pH 9.6
Blocking solution	3% BSA in PBS
Phosphate Citrate buffer (0.1M)	25.7 ml 0.2 M Na_2HPO_4 24.3 ml 0.1 M Citric acid pH 5
Phosphatase substrate solution	1 Tablet. Phosphatase Substrate 20 ml coating buffer 0.1 mM $MgCl_2$
Stopping solution	for AP-conjugate: 0.1 M EDTA for POX conjugate: 1M H_2SO_4
TMB-Substrate solution	1 Tablet. TMB 5 ml H_2O 0.05 M Phosphat-Citrat-Puffer pH 5 0.006% H_2O_2
Wash buffer	PBS/0.025% Tween

6.10.8 Antibodies

His Antibodies	Qiagen, Hilden
24-4 monoclonal against chitinase	Dept. of Molecular Parasitology
Goat anti-mouse IgG – AP	Dianova, Hamburg
Goat anti-mouse IgG –FITC	Dianova, Hamburg

6.11 Immunization of *Meriones uniguiculatus*

STP Adjuvant	0.4% Tween 20 1% Synperonic 10% Squalane in PBS
Alum Adjuvant	Reheis, Dublin, Ireland
Xylazin	Bayer Vital GmbH, Leverkusen
Ketamin	Pharmacia GmbH, Karlsruhe

7 ABBREVIATIONS

A	Adenosine
Ab	Antibody
A. viteae	*Acanthocheilonema viteae*
APS	Ammonium peroxidisulfate
APOC	African Programme for Onchocerciasis Control

Amp	Ampicillin
AP	Alkaline phosphatase
bp	Base pairs
BCA	2,2´Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyphosphate disodium salt
BSA	Bovine serum albumin (Fraction V)
cDNA	Complementary DNA
C	Celsius
C. elegans	*Caenorhabditis elegans*
C_t	Threshold cycle
CTAB	Cetyltrimethylamoniumbromide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
d.p.i	Days post infection
E. coli	*Escherichia coli*
EDTA	Ethylenediamino tetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
EtOH	Ethanol
Fig.	Figure
FCS	Fetal Calf Serum
GFP	Green Fluorescent protein
γ-rays	Gamma irradiation
h	Hours
HPLC	High performance liquid chromatography
hsp/HSP	Heat Shock Protein
Ig	Immunoglobulin
IPTG	Isopropyl-thio-ß-D-galactopyranoside
IU	International unit
kb	Kilo base
L1	First stage larvae
L2	Second stage larvae
L3	Third stage larvae
L4	Fourth stage larvae

LB	Luria Bertani
m	Milli
M	Molar
μ	Micro
2-ME	ß-Mercaptoethanol
mAb	Monoclonal antibody
Mf	Microfilariae
mRNA	Messenger RNA
NBT	Nitroblue tetrazoliumchloride
NC	Nitrocellulose
OCP	Onchocerciasis Control Program
OD	Optical density
ON	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pha	Pharynx development abnormal
PIPES	1, 4-Piperazinediethane sulfonic acid
PMSF	Phenylmethylsulphonylflouride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SDS	Sodium dodecylsulfate
TAE	Tris-acetate-EDTA
TB	Transformation buffer
TBS	Tris-buffered saline
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	2-Amino-2-hydroxymethyl-1,3-propandiol
U	Units
unc	Uncordinated
UV	Ultraviolet
V	Volt
X-gal	5-Bromo-4-chloro-3-indoxyl-ß-D-galactoside

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